# Freshwater Sponges and their Interaction with Bacteria Through Filtration, Retention and Antimicrobial Properties

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I confirm that the word limit is less than 100,000

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**Abstract**

As filterfeeders, freshwater sponges encounter bacteria in streams, rivers and lakes including those from faecal sources like enterococci and coliforms, which can exhibit antibiotic resistance with potential clinical impacts through e.g. infection of humans from recreational use of these environments.

Filterfeeding trials verified the potential of Irish freshwater sponges *Ephydatia fluviatilis* and *Spongilla lacustris*, which occupy wide ranges in the northern hemisphere, to reduce the abundance of *Escherichia coli* in ambient water. Plate counts of bacterial abundance were more reliable than monitoring methods involving turbidity or fluorescence measurements. Laboratory and field studies tested the application of the sponges for biomonitoring of microbial water quality. In the laboratory both sponge species retained *Enterococcus faecalis* in lower abundances than *E. coli*. Although gradual changes of abundance of enterococci and coliforms in sponge samples were also observed along a longitudinal river reach transect, the between river differences in retention of enterococci and coliforms were greater than within a single river.

The sponges' potential for facilitating conjugal antibiotic resistance transfer was explored in microcosms with *E. faecalis strains* resistant to either vancomycin or rifampicin. Lack of a significant difference between transconjugant numbers on double selection plates from microcosms with live or dead sponges suggested that filtration activity had no decisive role in conjugal transfer of monitored resistance traits.

Sponge gemmule surfaces were found to be associated with bacteria resistant to ampicillin, erythromycin, rifampicin, tetracycline, trimethoprim and vancomycin. Methanol extracts from freshwater sponges inhibited the growth of some nosocomial bacteria, with adult sponge extracts having a higher inhibitory effect than extracts from gemmule-grown sponges, indicating the contribution of the sponge microbiome. The antimicrobial properties of sponge samples varied with collection site, and the combination of sponge extracts from several sites caused the better inhibitor to become diluted and less effective as an antimicrobial agent.

**Abbreviations**

| | |
|---|---|
| ANOVA | Analysis of variance |
| ARB | Antibiotic resistant bacteria |
| ARG | Antibiotic resistance genes |
| A.U. | Arbitrary units |
| BMWP | Biological monitoring working party |
| cDNA | Complementary deoxyribonucleic acid |
| cfu | Colony forming units |
| COD | Chemical oxygen demand |
| *df* | Degrees of freedom |
| DGGE | Denaturing gradient gel electrophoresis |
| DMSO | Dimethyl sulfoxide |
| DMSP | Dimethylsufoniopropionate |
| DNA | Deoxyribonucleic acid |
| DO | Dissolved oxygen |
| DOC | Dissolved organic carbon |
| FAU | Formazin attenuation units |
| FISH | Fluorescent in situ hybridisation |
| GFP | Green fluorescent protein |
| HGT | Horizontal gene transfer |
| LPS | Lipopolysaccharides |
| LSD | Least significant difference |
| MIC | Minimal inhibitory concentration |
| MIZ | Minimal inhibitory zone |
| MPN | Most probable number |
| mRNA | Messenger ribonucleic acid |
| MRSA | Methicillin resistant *Staphylococcus aureus* |
| NLR | Nucleotide-binding domain and Leucine-rich repeat containing genes |
| PBS | Phosphate buffered saline |
| PCR | Polymerase chain reaction |
| qPCR | Quantitative polymerase chain reaction |
| rRNA | Ribosomal ribonucleic acid |
| SE | Standard error |
| SPSS | Statistical package for social sciences |

| | |
|---|---|
| SS/MS | Sum of squares/ Mean square |
| T/D | Transconjugant/Donor ratio |
| *Taq* | *Thermus aqualicus* |
| TMTC | Too many to count |
| TSA | Tryptone soya agar |
| TSB | Tryptone soya broth |
| UV | Ultraviolet |
| VBNC | Viable but not culturable |
| VRE | Vancomycin resistant enterococci |
| WWTP | Wastewater treatment plant |

**Statement of Original Authorship**

I hereby certify that the submitted work is my original work and was completed as a candidate for the degree stated on the title page. I have not obtained a degree elsewhere based on the research presented in this submitted work.

Allison Cartwright

Date

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# 1. Introduction

## 1.1 Project Rationale

Bacteria are vital components in the lifecycles of plants and animals. However, there are concerns over the multidrug resistance exhibited by a number of pathogenic bacteria which pose a serious risk to human health (Maragakis & Perl 2008; Yang *et al.* 2017). There is also concern that antibiotic resistant bacteria (ARB) from environmental reservoirs could infect humans thus passing to the clinic environment (Aminov 2009; Berendonk *et al.* 2015). Animals which interact with bacteria in an aquatic ecosystem include filterfeeding organisms. These organisms rely on small suspended particles in the water, including bacteria, for food. Freshwater sponges were chosen to test specific features in the interaction between bacteria and filterfeeders. They have two main interactions with bacteria through feeding and symbiosis. Their symbiotic community also contains ARB (Selvin *et al.* 2009). Although knowledge of marine sponge-bacteria processes has increased, their symbiotic bacteria are still a relatively unexplored area of research despite established knowledge on sponge feeding. Freshwater sponges also feed on bacteria and contain symbionts, but these symbionts have not been tested for antibiotic resistance.

The interactions between bacteria and freshwater sponges are poorly studied in comparison to marine sponges, probably due to the seasonality of their lifecycle. Freshwater sponges produce specialised structures called gemmules which allow the organism to die back at temperatures outside of their optimal 4-30 ℃ range. This suggests that the bacteria in sponges may also be limited to their host's active lifecycle stages, but this still needs to be tested. It is also unclear if gemmules are associated with ARB which are of clinical concern. Lupo *et al.* (2012) suggested that conjugal transfer between bacteria could occur in filterfeeders such as sponges, further highlighting the need to understand if sponges contain ARB and if sponges aid bacterial conjugal transfer. Finally, given the problem with antibiotic resistance and the potential of a 'post-antibiotic' era (Kenny *et al.* 2015), there is also a need to find new sources of antimicrobial agents. An immune system or antimicrobial compounds are necessary to protect host organisms. As marine sponges demonstrate a basic immune response and antimicrobial properties, the inhibitory effects of cosmopolitan freshwater sponges also need to be tested.

Further understanding of the pollution from certain bacteria in water is needed for better decision-making on water quality. Standard monitoring of microbial water quality uses spot sampling which could miss pathogenic bacteria which may have been in the water

immediately prior to the sample collection. As freshwater sponges continuously collect bacteria from the water, they could be used to extend the effective monitoring period for bacteria in water, but this has not been tested. As aquatic bacteria move through sponges, they can still be detected with analytical methods. Interactions with bacteria have mainly been studied on marine sponges, so research on freshwater sponges is needed. The use of sponges for sampling bacteria has potential to be developed to monitor pollution released from wastewater treatment plants (WWTP) and the quality of drinking water. It could also be expanded to sample for ARB.

This thesis aims to address the highlighted gaps identified above.

## 1.2 Thesis structure

The unifying theme of this thesis is the interactions of freshwater sponges with bacteria. As filterfeeders, they move bacteria through their bodies where they may be used for food (Chapter 3) or retained as symbionts, often for an unknown purpose. The abundance of bacteria in sponges allows them to act as a biosampler to identify bacterial groups from the river – both ARB or indicators of faecal contamination (Chapter 4). It therefore, follows that sponges are likely candidates to facilitate antibiotic resistant conjugal transfer between bacteria, because these are abundant within a confined space making the collision of bacteria possible (Chapter 5). Due to the interconnectivity of sponges with bacteria and their seasonal nature, it is also likely that sponges incorporate bacteria, as has been demonstrated with algae, into the gemmules for release upon hatching (Chapter 6). The above chapters investigate the association of sponges with bacteria, but to prevent infection, these hosts also need to exhibit antimicrobial properties (Chapter 7).

## 1.3 Aims, objectives and hypotheses

There are five experimental chapters in this research project. The first of these experimental chapters (Chapter 3) is focused on the following aims: a) investigate the ability of freshwater sponges to filter bacteria from the water and b) to determine different methods to quantify the changes in bacterial abundance.

The project hypotheses within chapter 3 are:

- H3.1: Sponges reduce bacteria abundance in water

- H3.2: Changes in bacteria abundance can be measured using agar plate counts, fluorescent intensity, and flow cytometry

The following two objectives have been developed:

1. Monitoring of *Escherichia coli* abundance in water with and without sponges (H3.1).
2. Detection of bacteria in water by plate counts, fluorescence intensity and flow cytometry (H3.2).

The second experimental component (Chapter 4) is focused on examining the ability of sponges to retain bacteria from the water thereby indicating microbial water quality. This section was completed in both laboratory and field trials.

The project hypotheses within chapter 4 are:

- H4.1: Sponges retain both coliforms and enterococci
- H4.2: Sponges can be used to monitor the microbial water quality in rivers

The following objectives were pursued with laboratory trials:

1. Investigation of the sponge's ability to retain *E. coli* and *E. faecalis* (H4.1, H4.2).
2. Comparison of bacterial retention by sponges with different relative abundances of *E. coli* and *E. faecalis* (H4.1).
3. Comparison of bacterial retention by sponges with different exposure time to *E. coli* and *E. faecalis* (H4.1).

Field investigations aimed to achieve the subsequent objectives:

4. Investigation of the variability of bacterial abundance in sponges within and between sites, in consideration of their proximity to point source pollution (H4.2).
5. Comparison of the concentration of coliforms and enterococci in sponge and water samples (H4.2).

The third experimental component (Chapter 5) investigates the role of freshwater sponges to facilitate conjugal antibiotic resistance transfer between bacteria.

The project hypotheses for chapter 5 are:

- H5.1: Sponges affect the transfer of antibiotic resistance between bacteria in the surrounding environment.
- H5.2: Sponges facilitate the transfer of antibiotic resistance between bacteria by filtration.

The following objectives were developed:

1. Assessment of effects of sponges on transconjugant numbers in their ambient environment (H5.1).
2. Comparison of transconjugant numbers in live and dead sponges to assess the impact of active filtration (H5.2).

The fourth experimental component (Chapter 6) investigates if gemmules contain ARB.

The project hypotheses for chapter 6 are:

- H6.1: Gemmules are associated with bacteria which are released upon hatching
- H6.2: Gemmules are associated with antibiotic resistant bacteria

The following two objectives were developed:

1. Investigation of bacteria release from gemmules upon hatching (H6.1).
2. Assessment of resistance to antibiotics of isolates from newly hatched sponges (H6.2).

The final experimental section (Chapter 7) tests if freshwater sponge extracts have an inhibitory effect on the growth of selected bacteria.

The project hypotheses within chapter 7 are:

- H7.1: Sponges inhibit the growth of bacteria.
- H7.2: Wild sponges with a fully developed symbiotic community show greater inhibitory effects than gemmule-grown sponges.
- H7.3: The inhibitory effect of sponge extracts will be comparable to other antimicrobial agents.

- H7.4: Mixing of sponge extracts will enhance their antimicrobial properties.
- H7.5: Sponges from rivers with higher bacteria loads show greater inhibitory effect.

The following objectives were developed:

1. Identification of the existence of antimicrobial effects in extracts of freshwater sponge tissue through growth inhibition tests on selected bacterial strains (H7.1).
2. Comparison of the inhibitory effect of sponge extracts between laboratory-reared sponges grown from gemmules with a low diversity microbiome and adult wild sponges with a high diversity microbiome (H7.2).
3. Comparison of bacterial growth inhibition by sponge extracts to those by an established antimicrobial plant extract and an inorganic chemical substance with antimicrobial properties (H7.3).
4. Comparison of the bacterial growth inhibition by sponge extracts from different rivers and mixtures of these extracts (H7.4).
5. Comparison of the bacterial growth inhibition of sponge extracts from different sites and sponge species in individual rivers (H7.5).

## 1.4 Chapter overview

Chapter 1 – Outlines the project rationale, aims, objectives, hypotheses and the information contained in each chapter.

Chapter 2 – Contains a literature review on the current knowledge for all experimental chapters and provides background to the research completed on sponges in related topics to this thesis.

Chapter 3 – Contains information on sponge filtering to remove bacteria from water. By using GFP tagged *E. coli* it was possible to quantify changes in bacterial abundance using agar counts and fluorescent methods including flow cytometry and fluorospectrometry.

Chapter 4 – Investigates the retention of bacteria by freshwater sponges. Laboratory trials tested the retention of *E. coli* and *E. faecalis* by sponges with variation in bacterial loading. Field trials quantified selected bacteria in sponges and how this related to aquatic loads.

Chapter 5 – Investigates if sponges are associated with the antibiotic resistance conjugal transfer between bacteria. Also demonstrates that sponges affect the ARB in the surrounding water.

Chapter 6 – Shows surface-disinfection of gemmules with hydrogen peroxide did not remove all bacteria. The bacteria on the gemmule surface show antibiotic resistance.

Chapter 7 – Identifies the antimicrobial effects of sponges against selected nosocomial bacteria. This ability varies with sponge species, and collection site.

Chapter 8 – Discusses the main experimental findings of the thesis and the unifying themes. It also identified the limitations and made recommendations for future work.

Chapter 9 – Concludes the research findings of this thesis

Appendix – Contains information on sponge distribution and the method used for hatching gemmules.

References – Contains details of the literature cited in this thesis.

# 2. Literature review

This review outlines the basic structures of sponges, introduces freshwater sponges, their gemmules and where they are found in Ireland. The antibiotic resistant properties of bacteria with reference to *E. coli* and *E. faecalis* will then be discussed. The interactions of sponges and bacteria will be explored by addressing how sponges filterfeed, retain bacteria and their relationship with symbiotic bacteria. The process of conjugal transfer and its occurrence in organisms will also be discussed. Finally, selected literature on the antimicrobial properties of sponges will be explored. Most of the literature found was for marine sponges with a limited number involving freshwater sponges.

## 2.1 Introduction to sponges

Sponges/Porifera are considered to be the simplest type of metazoan organism (Karlep *et al.* 2013). Sponges are primarily marine, with 8,500 known species but only 200 (2%) have colonized into freshwater habitats (Itskovich *et al.* 2013). All freshwater sponges belong to the order Spongillina in the class Demospongiae (Morrow & Cárdenas 2015) which are characterized by siliceous spicules and spongin fibres, a form of collagen (Ackers *et al.* 2007). Spicules are glass-like structures which are present on the sponge's surface to provide protection and support (Ackers *et al.* 2007).

All sponges are filterfeeders (Figure 2.1). Water enters a sponge colony through small pores, the ostia, and leaves through a large central pore, the osculum. The water exits the osculum at high velocity, creating a negative pressure within the sponge, which draws in more water (Brusca & Brusca 2003) and causes the movement of food particles into the sponge. Sponges have four main types of cells: choanocytes which line the central water-filled cavity called the spongocoel within the sponge and beat their flagella to generate water movement for suspension feeding; pinacocytes, flattened cells that can contract and expand to maintain the sponge shape and structure; amoebocytes which store food, eliminate waste products and produce the spicules; and porocytes through which the water and particles enter the sponge (Brusca & Brusca 2003). These cells are attached to a connective tissue, the mesohyl, which makes up the sponge's main body. Symbiotic bacteria and algae are also found in sponges, occurring throughout the body of marine sponges, but they are limited to vacuoles in freshwater sponges (Gernert *et al.* 2005).

The spicules which make up the sponge skeleton are also used for their identification. Freshwater sponges can rarely be identified by gross morphology and colour, as these show high variability within an individual species, and many species look similar (Ackers

et al 2007). Spicules, however, vary with each species of sponge (Ackers et al 2007). There are two main types of spicules in the adult sponge: megascleres which form the main skeleton, and microscleres which add support to the skeleton (Paduano & Fell 1997). Freshwater species and a few marine species also have a third type of spicule, gemmoscleres that are entirely unique to each species (Paduano & Fell 1997). These protect the gemmules (Figure 2.3). Freshwater sponges are primarily identified by their microscleres and gemmules due to the similarity of megascleres in all species (Manconi & Pronzato 2002). If gemmoscleres are not present, DNA analysis is needed for identification. Figure 2.2 shows examples of the spicules found in two different freshwater sponge species: *Ephydatia muelleri* and *Spongilla lacustris*.

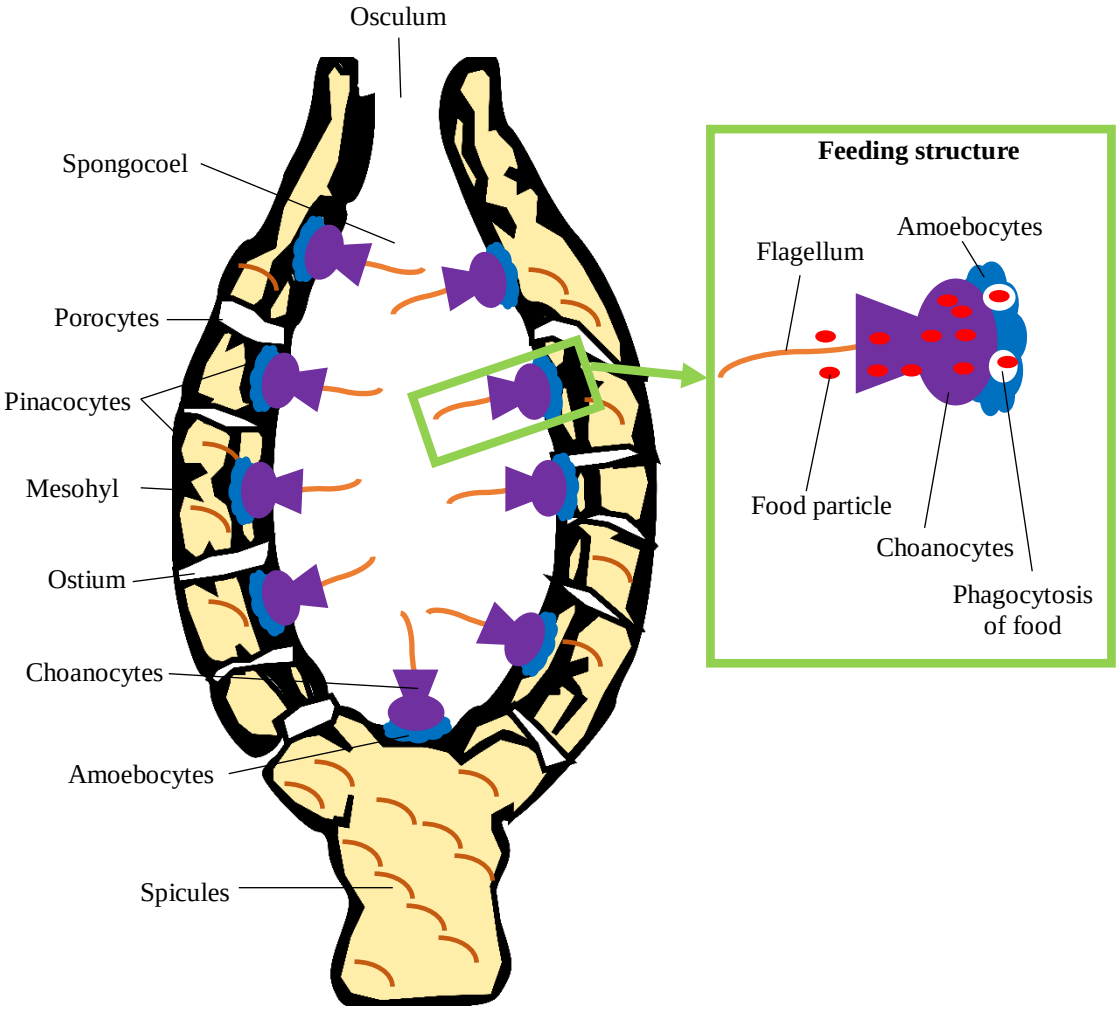



Figure 2.1. The general structure of a sponge. Adapted from Cummings (2014).

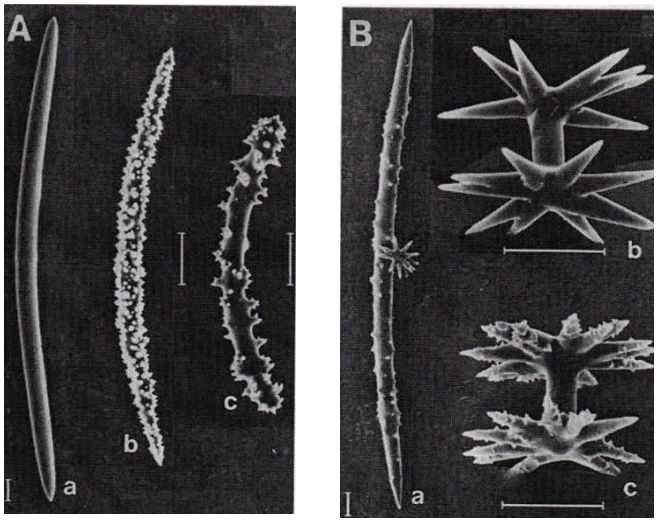



Figure 2.2 An example of the spicules found in two freshwater sponges for (A) *Spongilla lacustris* where a = megasclere, b = microsclere and c = gemmosclere. (B) *Ephydatia muelleri* were a = megasclere, b = smooth gemmosclere and c = spined gemmosclere. The scale bar = 10 µm. From Økland & Økland (1996).

Freshwater sponges create a special structure called gemmules which allows the sponges to die back in unfavourable conditions and re-grow when conditions improve (Simpson & Fell 1974; Paduano & Fell 1997). These gemmules contain totipotent cells within a tough chitinous layer coated with the gemmoscleres (Figure 2.3) from which an adult sponge can be grown (Manconi & Pronzato 2002). Most sponges found at higher latitudes die back in winter and re-grow from their gemmules in the spring. Sponges can spread in geographical distribution through budding and sexual reproduction, but gemmules are likely to be more important for extending their geographic distribution from a source population in one water body to an unconnected aquatic environment (Gugel 2001; Cocchiglia *et al.* 2013; Itskovich *et al.* 2013). Gemmules even remain intact after passing through the digestive system of water birds, thus, allowing migratory birds to transport gemmules to more distant waterbodies (Paduano & Fell 1997).

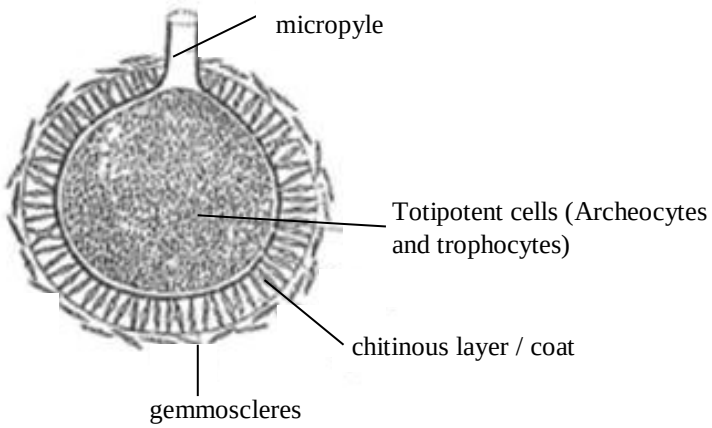



Figure 2.3. Structure of a freshwater sponge gemmule. Adapted from: Annandale (1911).

Once a sponge has formed gemmules, these have high resistance to challenging environmental conditions including desiccation and temperatures below freezing (Barbeau *et al.* 1989; Fell & Bazer 1990). For example, *Racekiela ryderi* gemmules are able to survive desiccation for five months and exposure to -20 ºC for a month (Fell & Bazer 1990). The level of resistance, however, appears to vary between sponge species; for example, *E. muelleri* gemmules are more resistant to low temperature than *R. ryderi* (Barbeau *et al.* 1989) and *S. lacustris* has no resistance to desiccation (Fell & Bazer 1990). Gemmules are also resistant to exposure to chemicals e.g. hydrogen peroxide. Hence the gemmule surface can be cleaned and disinfected to remove pollutants and microorganisms from exterior surfaces before hatching sponges for laboratory experiments (Rasmont 1970; Reiswig & Miller 1998). During gemmule formation, sponges incorporate algae from the adult sponge into the gemmule (Simpson & Fell 1974; Willianson & Williamson 1979). However, it remains unclear if gemmules also contain bacteria from the adult sponge.

## 2.2 Freshwater sponges in Ireland

In Europe 14 species of freshwater sponges have been recorded (Økland & Økland 1996). Five of these sponge species occur in Ireland: *Ephydatia fluviatilis, R. ryderi* (previously *Anhetermeyenia ryderi* and *Hetermeyenia ryderi*), *S. lacustris, E. muelleri* and *Eunapius fragilis* (previously *Spongilla fragilis*). These species are from the family Spongillidae and are found throughout the northern hemisphere and considered cosmopolitan species. Due to this wide geographic range, there has been some research on them, but usually, the number of studies in a particular region is very limited. Ireland's freshwater sponges are poorly studied and little research has been conducted since the early 1900s (Cocchiglia *et al.* 2013) with only Stephens (1919) having studied regions in Northern Ireland as they are not considered a priority group for study.

In Ireland, *E. fluviatilis* was found in more sites in Ireland than other species by Stephens (1919) and Lucey & Cocchiglia (2014). *E. fluviatilis* was typically recorded in rivers but was also found in lakes. *S. lacustris* was also frequently recorded in both studies but was more abundant in lakes than in rivers. Species richness of sponges typically peaks in the zone where a lake flows out into the river, as species with preference for both rivers and lakes are likely to occur here (Stephen, 1919; Økland & Økland 1996).

While proximity to lakes is a likely prerequisite for the existence of some sponge species, another factor affecting sponge distribution appears to be the availability of hard substrate for attachment (Paduano & Fell 1997). Of the sponges found in Ireland, only *S. lacustris* occurs on soft sediment where it grows colonies with finger-like projections, while all other sponges require a hard substrate for growth (Stephens 1919). Turbid water and siltation can also restrict sponge growth by blocking the choanocytes in the sponge, reducing its ability to feed and causing death to the organism if exposure is long-term (Whalan *et al.* 2007). Sponges have a high tolerance to variations in temperature, electrical conductivity, pH, water colour, hardness ($Ca^{2+}$and $Mg^{2+}$) and silica content where they can survive in water with conductivity of 4-304 µS $cm^{-1}$, pH of 4.2-9.6 and calcium concentrations of 0.7-56.8 mg $l^{-1}$ (Økland & Økland 1996). This has led several investigators to conclude that chemical properties of the water are unlikely to affect sponge distribution (Økland & Økland 1996; Lucey & Cocchiglia 2014).

Many freshwater sponges have a symbiotic relationship with algae like *Chlorella* species (Frost & Williamson 1980). These algae appear vital for sun-exposed sponges as they can protect the sponge cells from membrane damage by ultraviolet (UV) radiation and aid the sponge growth by providing supplementary nutrition (Frost & Williamson 1980; Wilkinson 1980). Sponges without symbiotic algae have been found to die if exposed to UV light (Wilkinson 1980) but the presence of specimens with algae is limited to the photic zone. In combination, this can lead to zonation in aquatic environments where, for example, green sponges live in the sun-exposed locations of a river, while asymbiotic sponges grow in shaded areas.

### 2.3 Bacteria and antibiotic resistance

Aquatic ecosystems are one of the main reservoirs for ARB (Wright 2010; Marti *et al.* 2014). Within the aquatic ecosystem, there are hotspots for ARB including at the outflow points from WWTP and from farm runoff (Berendonk *et al.* 2015). Both of these are ARB hotspots because they combine antibiotics, mostly unmetabolized and excreted, at a diluted concentration in wastewater, with the bacteria from humans and farm animals (Michael *et al.* 2013). Selection pressure facilitates the survival and spread of resistant organisms. Over time, the level of resistance can increase to allow the bacteria to withstand higher doses of the antibiotic (Dzidic & Bedekovic 2003). Although resistance can naturally occur, the misuse of antibiotics within a clinical and veterinary setting has

enhanced selective pressure for ARB making antibiotics less effective (Lupo *et al.* 2012; Saurav *et al.* 2016).

Once ARB were created, they can transfer the resistance genes to other bacteria in a process known as Horizontal Gene Transfer (HGT). There are three major types of HGT in bacteria: transformation, transduction, and conjugation. Transformation is where free DNA fragments can be incorporated into other bacteria (Wilson & Salyers 2011). Transduction occurs when DNA is transferred using a bacteriophage, and conjugation occurs where DNA is transferred using direct contact between cells (Wilson & Salyers 2011). The mechanism of conjugation is different between Gram-positive and Gram-negative bacteria. In Gram-positive bacteria conjugation occurs between bacteria through direct contact, but in Gram-negative bacteria, the cells have to form a connection (pilus) before conjugation occurs (Massoudieh *et al.* 2007). The size of genetic material which can transfer between bacteria varies with each mechanism. Conjugation can transfer the largest amounts of DNA with van Schaik *et al.* (2010) finding multiple plasmids in *Enterococcus faecium* obtained through conjugal transfer that were over 50 kb in size with some as large as 240 kb. Transformation is the mechanism that transfers the least information with T-DNA of 1-14 bp being successfully transformed into the fungal cells of *Fusarium oxysporum* (Mullins *et al.* 2001).

The transfer of information can include antibiotic resistance genes (ARG) which potentially impact on human and animal health (Aminov & Mackie 2007; Flores Ribeiro *et al.* 2014). Enterococci, for example, have emerged as common pathogens since the 1970s and in the 1990s, they showed high susceptibility to vancomycin and ampicillin with 90% and 85% of isolates killed (Reacher 2000; Sandoe *et al.* 2003). However, enterococci have since acquired resistance plasmids which exhibit vancomycin resistance and so vancomycin resistant enterococci (VRE) accounted for 8.5-10.8% of the enterococci infections in the UK and infection from this bacteria group is rising around the world (Brown *et al.* 2008; Emaneini *et al.* 2016). Resistance is not only increasing in enterococci, and in 2016, a woman died after contracting *Klebsiella* sp. resistant to all known antibiotics which prevented treatment (Chen *et al.* 2017). The bacterium was resistant to over 26 antibiotics and although it was tested for the presence of the New Delhi metallo-beta-lactamase enzyme, it was not tested for resistance plasmids. However, it is possible that this bacterium contained some plasmid bound resistance and genes acquired from horizontal transfer. Unless new antibiotics are found, we face a post-

antibiotic era where once again, bacterial infections will not be controlled (Kenny *et al.* 2015).

There are many bacteria which exhibit multidrug resistance, but only enterococci and coliforms will be discussed below as they are the prime focus of this thesis. Enterococci are Gram-positive bacteria usually found in the oral cavity and gastrointestinal tract (Jett *et al.* 1994). They are found in warm blooded animals and leave their bodies in faeces where they can pollute water (Jett *et al.* 1994; Ghosh *et al.* 2011; Novais *et al.* 2013). They are opportunistic pathogens of humans. The most common infections from enterococci are urinary tract infections (Jett *et al.* 1994; Simonsen *et al.* 2003). Most enterococci infections are from *Enterococcus faecalis* or *E. faecium*. These bacteria have been associated with infection since the 1970s with most infections from *E. faecalis* in the 1990s, but *E. faecium* has since become a more common cause of infection (Jett *et al.* 1994; Arias & Murray 2012). Individual strains of enterococci can be resistant to many antibiotics (Doud *et al.* 2014). Resistance to antibiotics includes ciprofloxacin, erythromycin, rifampicin, and vancomycin (Arvantidou *et al.* 2001; Arias & Murray 2012). Enterococci developed resistance through natural mutation, transduction or the HGT of resistance genes (Lupo *et al.* 2012; Conwell *et al.* 2017). Many enterococci also contain integrons, gene cassettes and plasmids which are highly mobile between cells (Jett *et al.* 1994; Zhang *et al.* 2009). They also have general protection mechanisms against antibiotics e.g. efflux pumps. Efflux pumps are transmembrane proteins which bacteria use to remove high concentrations of antibiotics from their cells thus preventing toxicity and death (Lee *et al.* 2003). There are many types of efflux pumps in bacteria which mostly help bacteria to cope with toxic drugs (Mahmood *et al.* 2016). There are a number of specific efflux pumps genes which can also vary with bacterial species, in *Pseudomonas aeruginosa*, for example, there are estimated to be 12 pumps including MexAB-OprM (Mahmood *et al.* 2016). In four South African Rivers, efflux pump genes including *tetL* and *msrC* were found in 21 of the 124 enterococci tested (Molale & Bezuidenhout 2016). The genes for efflux pumps can be plasmid bound and thus transfer between bacteria, improving their tolerance to antibiotics.

Enterobacteriaceae are a large group of Gram-negative bacteria which contains bacterial species including *Salmonella, Klebsiella* and *E. coli*. They are usually found in the gastrointestinal tracts of animals and humans (Yang *et al.* 2017). This means they are also released in faeces where they can enter the water (Harwood *et al.* 2014). Many of the

bacterial strains which comprise Enterobacteriaceae are harmless, but some are pathogenic. One strain, *E. coli* 0157: H7, for example, is of concern within the food industry because it is widely contained in cattle faeces where it can infect food products either through direct contact, or contact with irrigation water (Solomon *et al.* 2002). *E. coli* can cause opportunistic infections including urinary tract infections, septicaemia and pneumonia (Mahmood *et al.* 2016). *E. coli* shows resistance to antibiotics including streptomycin, gentamicin, trimethoprim, and carbapenem (Jacoby 2009; Yang *et al.* 2017). The multidrug resistance traits observed in *E. coli* are similar to those highlighted above in enterococci and include integrons gene cassettes, efflux pumps, gene mutation and the acquisition of resistance genes or plasmids from HGT (Imuta *et al.* 2008; Davies & Davies 2010; Phornphisutthimas *et al.* 2007; Yang *et al.* 2017). *E. coli* can contain efflux pump genes such as AcrAB-TolC and, integrons including *addA1* and *dfrA1-sat2-aadA1* (Mahmood *et al.* 2016; Yang *et al.* 2017). The latter were isolated from animal faeces showing their presence within organisms and their potential to enter the aquatic environment from surface runoff.

2.4 Bacteria filtering capacity of sponges for feeding

Sponges are important for filtering water, with a sponge covering 10 $cm^2$ surface area estimated to filter more than 125 l of water a day (Frost 1980). While they are filtering the water column, they feed on organic matter, phytoplankton and bacteria (Longo *et al.* 2010). It has been estimated that sponges consume up to 80% of the filtered matter (Stabili *et al.* 2008) and retain a high number of the particles, because water comes into contact with the majority of their body structure (Hill & Hill 2002). This means that they have an important role in the benthopelagic coupling where they filter food and nutrients out of the water for use within the benthos (Gifford *et al.* 2007; Longo *et al.* 2010).

Sponges are unselective filterfeeders, but the size of their ostia, the inhalant pores, restricts them to feeding on particles smaller than 50 µm (Figure 2.1). Once particles enter the sponge, they are sorted for digestion based on size. Larger particles such as aggregated bacteria and algae fall within the size range of 5-50 µm and are therefore digested by phagocytosis within the endopinacocytes, which are internal cells supporting the sponge structure (Francis & Poirrier 1986; Vohmann *et al.* 2009). Single cells of bacteria fall within the size range of 0.1-5 µm and are digested by phagocytosis in food trapping cells called choanocytes (Francis & Poirrier 1986), making sponges one of the benthic organisms capable of utilising these single bacteria cells (Reiswig 1975; Willenz *et al.*

1986). Regardless of size, digestion and the subsequent movement of digested particles takes place in amoebocytes which are used for transport and digestion of cells (Gernert *et al.* 2005).

Only the feeding of sponges on bacteria will be considered further in this review with most research focusing on the rate at which this occurred. These studies have included laboratory and field experiments to quantify the ability of sponges (usually marine species) to filter different bacterial species including *E. coli* (Willenz *et al.* 1986; Fu *et al.* 2006). Laboratory experiments usually involved exposing wild sponges to controlled quantities of individual bacteria species, while field experiments sampled the bacteria in the sponges and compared them to the surrounding water by taxonomic identification. Methods to quantify the bacteria removal by sponges include direct plating onto agar (Reiswig 1975; Milanese *et al.* 2003; Fu *et al.* 2006), using particle counters (Frost 1980; Francis & Poirrier 1986), microtiter plates (Willenz *et al.* 1986), fluorescence microscopy (Wehrl *et al.* 2007) and flow cytometry (Topçu *et al.* 2010; Perea-Blázquez *et al.* 2013).

Regardless of the method choice, all studies demonstrated that sponges removed bacteria from the water through feeding (Table 2.1a). Laboratory feeding trials with marine sponges found filtration rates ranging from $7 \times 10^6$ cells $h^{-1}$ $cm^{-3}$ to $1.74 \times 10^6 – 2.76 \times 10^6$ g $h^{-1}$ of bacteria (Milanese *et al.* 2003; Wehrl *et al.* 2007). However, due to a lack of standard methods or calculations, it is difficult to compare these rates. The filtration rate is also affected by the health of the sponge as well-fed sponges exhibit higher filtration rates during subsequent experiments than those starved before experiments with maximal filtration of 1.5 MPN $g^{-1}$ of coliforms by well-fed sponges but no observed retention in starved sponges (Longo *et al.* 2010). The type and shape of bacteria, however, do not seem to affect sponge filtering unless the bacteria are sponge symbionts. Sponges filtered bacteria species with a symbiotic relationships at a lower rate than other aquatic bacteria without a symbiotic relationship with sponges (Table 2.1a; Wehrl *et al.* 2007). The filtration rates of aquatic bacteria were in the magnitude of $10^6$ g $h^{-1}$ for seven bacteria species including *Vibrio* sp., *Bacillus* sp. and *Pseudoaltermonas* sp. but the sponge symbionts were only filtered at $10^6$ g $h^{-1}$. Although sponges did remove bacteria from water as they filter, some bacteria were released again, but at a vastly reduced quantity (Reiswig 1975; Wehrl *et al.* 2007).

Table 2.1a. Summary of laboratory studies focusing on the ability of marine sponges to filter bacteria, including the investigated sponge species and the main findings.

| **Species and study aim** | **Main findings** | **Authors** |
|---|---|---|
| Removal of *E. coli* and *Vibrio anguillarum* by *Hymeniacidon perlevis* | • Sponge retained 1.55 x $10^2$ *E. coli* cells $h^{-1}$ (clearance rate 30.5 – 32.2 ml $h^{-1}$).<br>• Not all *E. coli* were ingested by sponge cells (not quantified), some remained in sponge tissue | Fu *et al.* (2006) |
| Removal of bacteria by fed, or starved *H. perlevis* | • Fed sponges accumulated more bacteria than starved individuals.<br>• Maximum clearance rate: 59 ml $h^{-1}$. | Longo *et al.* (2010) |
| Bacteria clearance rate by *Chondrilla nucula* | • Retention of 6 -7 x $10^6$ cells $h^{-1}$ $cm^{-3}$<br>• 1 $m^2$ surface cover of *C. nucula* filtered 14 l $h^{-1}$ retaining 7.4 x $10^{10}$ cfu $h^{-1}$ *E. coli* | Milanese *et al.* (2003) |
| Feeding of *Haliclona permollis* on seawater bacteria | • Bacteria in exhalent water lower than inhalant water.<br>• Around 70% of bacteria removed | Reiswig (1975) |
| Bacteria uptake by *Aplysina aerophoba* | • Retention rate of specific bacteria spp.: 1.74 x $10^6$ – 2.76 x $10^6$ g $h^{-1}$<br>• Retention of sponge symbiotic species: 5.37 x $10^4$ g $h^{-1}$<br>• Bacteria structure and shape did not affect uptake | Wehrl *et al.* (2007) |

Little laboratory research has been conducted on the filtering effects of freshwater sponges on bacteria. All the studies found in June 2017 are summarized in Table 2.1b. Similarly to marine sponges, freshwater sponges also removed bacteria. The rate of filtration varied from 0.26 ml $s^{-1}$ to 2 ml $s^{-1}$ (Frost 1980; Francis & Poirrier 1986) while Longo *et al.* (2010) found marine sponges filtered 1 ml $s^{-1}$, so the filtration rates of the marine and freshwater sponges are comparable. Freshwater sponges showed high removal of *E. coli* from the water where 2 x $10^2$ cfu were removed by *E. fluviatilis* or *Spongilla alba* each day (Francis & Poirrier 1986). Bacteria appear to be an important food source for sponges, evidenced by sponges filtering bacteria in similar quantities irrespective of the presence of other food sources like algae or yeast (Frost 1980). When sponges were fed *Aerobacter aerogenes* they retained $10^5$ cells $ml^{-1}$ within an hour regardless of whether this formed the only food source, or was supplemented with the yeast *Rhofotorula glutinis* (Frost 1980).

Table 2.1b. Summary of laboratory studies focusing on the ability of freshwater sponges to filter bacteria, including the investigated sponge species and the main findings.

| **Species and study aim** | **Main findings** | **Authors** |
|---|---|---|
| *E. coli* filtering by *E. fluviatilis* | • *E. coli* of 1.0 µm and 1.9 µm were filtered at similar rates<br>• Clearance rate: 0.26 ml $s^{-1}$. | Francis & Poirrier (1986) |
| Bacteria filtering by *S. lacustris* on *Aerobacter aerogenes* with and without the addition of algae or yeast | • Removal rate increased with sponge size<br>• Maximum filtration efficiency at 23 ºC<br>• Bacteria removal remained similar irrespectively of the presence of yeast or algae - sponges selectively filtered bacteria<br>• Clearance rate up to 2 ml $s^{-1}$ | Frost (1980) |
| *S. lacustris* feeding on radioactively labelled *E. coli* | • *E. coli* in sponges increased for 24 h before feeding reduced the abundance<br>• Digestion of bacteria started immediately but was higher after 24 h | Willenz *et al.* (1986) |

Filtration studies typically involved the exposure of sponges to high quantities of a known species of bacteria over short periods of time <48 h followed by estimates of the removal of bacterial cells from the water. In an aquatic system, however, there are many bacteria and other organisms including the cyanobacteria species *Synechococcus* and *Prochlorococcus* available to the sponges for feeding, the diversity and abundance of which will change over time (Perea-Blázquez *et al.* 2013). Studies comparing the bacteria in water and sponges are summarized in Table 2.1c. Although heterotrophic bacteria are utilized for sponge feeding as they filter the water, they were not always the main diet of sponges as cyanobacteria and phytoplankton were occasionally consumed at a higher rate (Ribes *et al.* 1999; Topçu *et al.* 2010). Therefore, the efficiency of sponges to remove bacteria varied from 33 to 84% (Ribes *et al.* 1999; Pile *et al.* 1997) depending on their importance in the sponge's diet. The higher efficiencies occurred with freshwater sponges during the absence of water turbulence when a zone of depleted bacteria directly adjacent to the sponges was recorded (Pile *et al.* 1997). This could, therefore, indicate that in laboratory feeding trials the bacterial feeding rates were overestimated due to a zone of depletion adjacent to the sponge, which would not occur naturally with water mixing.

Overall, the studies did not have high replication. All of the papers cited in Tables 2.1a and 2.1b had fewer than five replicates, except for Reiswig (1975) who used 15 replicates. The bacteria removal by sponges also showed a high variation between individual

sponges with removal rates of 30-90% for planktonic bacteria (Reiswig 1975). This was especially noticeable in the results published by Reiswig (1975) due to the higher level of replication. Some sponges had episodes of 'negative bacterial removal' at times when results suggested a net release of bacteria cells (Reiswig 1975; Willenz *et al.* 1986; Milanese *et al.* 2003). The bacteria in sponges were also likely to be underestimated by plate counting methods as many bacteria species and injured or stressed bacteria, do not grow on agar (Reiswig 1975).

Table 2.1c. Summary of field studies for bacteria filtration by sponges including the investigated sponge species and the main findings (DOC = Dissolved Organic Carbon).

| **Species and study aim** | **Main findings** | **Authors** |
|---|---|---|
| Removal of DOC and bacteria by *Halisarca caerulea, Mycale microsigmatosa* and *Merlia normani* | • Removal of DOC exceeded that of bacteria by two orders of magnitude.<br>• It remained unclear if symbiotic bacteria or sponges utilise DOC. | De Goeij *et al.* (2008) |
| Removal and retention of bacteria and cyanobacteria by *Crella incrustans*, *Haliclona venustina* and *Strongylacidon* sp. | • Bacteria were the main food source (but there were seasonal variations where cyanobacteria consumption was higher in autumn)<br>• Bacteria abundance in water did not affect abundance in sponges. | Perea-Blázquez *et al.* (2013) |
| Feeding on plankton <5 µm by *Mycale lingua* | • Around 74% of filtered bacteria were consumed. | Pile *et al.* (1996) |
| Feeding on picoplankton by *Baikalospongia bacillifera* and *Baikalospongia intermedia* | • Reduction in bacteria concentration was 84% for *B. bacillifera* and 71% for *B. intermedia*.<br>• Filtration depleted bacteria in the water column up to 1 m above the lake bed. | Pile *et al.* (1997) |
| Plankton feeding rates of *Dysidae avara* | • Heterotrophic bacteria contributed 33 – 43% of the sponge's diet depending on the season. | Ribes *et al.* (1999) |
| Uptake and retention of picoplankton by *Spongia officinalis* | • Cyanobacteria represented the main food source; least consumed were heterotrophic bacteria.<br>• Sponges only removed 48% of heterotrophic bacteria. | Topçu *et al.* (2010) |
| *E. muelleri* feeding on pico and nanoplankton | • Sponges were fed on bacteria and algae of pico- and nanoplankton size<br>• Feeding was low during gemmule formation. | Vohmann *et al.* (2009) |

2. 5 Bacteria retention by sponges for bioremediation and pollution monitoring

As discussed above, bacteria can constitute the main food source for sponges, but sponges also have the ability to retain bacteria in their mesohyl (Wehrl *et al.* 2007; Stabili *et al.* 2008; Perea-Blázquez *et al.* 2013). A sponge tissue sample, therefore, contains bacteria acquired for feeding and those bacteria retained as part of the sponge's symbiotic community. Feeding and symbiosis together, remove bacteria from the water and offer bioremediation of bacterial pollution (Stabili *et al.* 2008), a service also offered by other filterfeeders e.g. mussels. In aquaculture systems the bioremediation of sponges, *H. perelevis* and the mussel, *Mytilus galloprovincialis* removed *Vibrio* sp. and *E. coli* from the water with maximal rates of $10^8$ and $10^5$ cfu $g^{-1}$ for sponges and mussels respectively (Longo *et al.* 2016). Sponges were also able to concentrate bacteria during the filtration process; hence the number of aquatic bacteria in the tissue of a filtering sponge was likely to exceed that of bacteria in a comparable volume of the ambient water (De Goeij *et al.* 2008; Stabili *et al.* 2008; Topçu *et al.* 2010). Stabili *et al.* (2008), for example, recorded faecal coliforms at the abundance of 1.2 MPN $g^{-1}$ from the sponge *Hymeniacidon perelevis* while these were 0.1 MPN $ml^{-1}$ in ambient water.

Marine sponges have been observed to contribute to the remediation of aquatic environments receiving sewage effluents (Longo *et al.* 2010). It has been estimated that sponges can accumulate 7 x $10^{10}$ of *E. coli* cells within each 1 $m^2$ of sponge surface, removing these bacteria from the water and thereby improve overall water quality (Milanese *et al.* 2003; Gifford *et al.* 2007). By sampling sponges for selected taxa of bacteria, sponges were used to monitor pollution, but it is difficult to know if these monitored bacteria were pollution indicators or naturally occurring. Some pollution indicator bacteria can also occur naturally in the sampled environments, e.g. coliforms and *Pseudomonas* spp. (Kefalas *et al.* 2003). Kefalas *et al.* (2003) analysed the bacteria found in the marine sponge *Spongilla officinalis* finding *E. coli*, *Pseudomonas* and *Aeromonas salmonicida* which originated from faecal sources and aquatic crustaceans and fish. *Pseudomonas*, for example, is found aquatically and it can be pathogenic to fish (Kefalas *et al.* 2003). Therefore, care needs to be taken when using bacteria as indicators of water contamination.

Using sponges for bioremediation or monitors of bacterial pollution is further complicated by factors affecting sponge filtration. During flow conditions where high amounts of suspended sediment are carried by the water, sponges are unlikely to be

filtering as they may contract upon physical contact with particulate matter exceeding the size of their food (Elliott & Leys 2007). Sponges can also stop filtering water at certain times in their lifecycle or when pollution is too high, reducing their removal of bacteria (Milanese *et al.* 2003). Therefore, they are not good indicators of severe pollution episodes or useful for bioremediation during storm events.

Several studies have indicated that sponges incorporated specific bacteria into their body as they filtered the water (Wehrl *et al.* 2007; Stabili *et al.* 2008; Perea-Blázquez *et al.* 2013). However, these studies only indicated the presence of bacteria in the water but did not investigate if the bacteria in sponges were related to the bacterial abundance in water. If the sponges represented the bacterial abundance in the water over a longer time-period, they could be used to monitor bacterial pollution events. However, this requires for sponges to be able to distinguish symbiotic bacteria from pollution indicator bacteria. This may be possible as sponges detect cellular surface molecules on bacteria. Gardères *et al.* (2015) found that the marine sponge *S. domuncula* detected specific LPS (lipopolysaccharides) from *Endozoicomonas, Pseudoalteromonas* and *E. coli* which were used for an immune response whereby sponges released macrophage genes in response to *E. coli*. This may indicate some selection in sponges where they can choose which bacteria are filtered.

2.6 Symbiotic bacteria in sponges

Symbiotic bacteria have been found in many different sponges including *E. fluviatilis, Fasciospongia cavernosa* and *Haliclona* sp. (Selvin *et al.* 2009; Costa *et al.* 2013; Hoppers *et al.* 2015) several studies have been solely focused on the identification of all symbiotic organisms which form the sponge microbiome and include the phyla Proteobacteria, Chlamydiae and species such as *Bacillus* spp. (Gernert *et al.* 2005; Costa *et al.* 2013; Eythorsdottir *et al.* 2016). Another current focus in the investigation of symbiotic bacteria in sponges is the attempt to find isolates with antibiotic resistance or isolates containing bioactive molecules which could eventually be used in a clinical setting to treat humans against bacterial infections (Dunlap *et al.* 2007; Pejin *et al.* 2014; Hoppers *et al.* 2015; Section 2.8).

For the identification of symbiotic bacteria in sponges, culture-based methods have largely been replaced by procedures which involve DNA extraction (Thacker & Freeman 2012). Generally, DNA extraction involved amplifying the 16S rRNA via the Polymerase

Chain Reaction (PCR; Gernert *et al.* 2005; Eythorsdottir *et al.* 2016). PCR products can then be used for denaturing gradient gel electrophoresis (DGGE) to isolate the product (Keller-Costa *et al.* 2014; Hoppers *et al.* 2015), or cloned for sequencing (Gernert *et al.* 2005; Eythorsdottir *et al.* 2016). Such studies have been able to identify different symbiotic bacteria species or provide a list of different phyla/classes of bacteria in a sponge.

Sponges collected from the field showed a wide range of different symbiotic bacteria from many phyla including the phyla Proteobacteria, Chlamydiae and Bacteroidetes (Table 2.2). The number of bacterial species, which were detected in sponge tissue without having a presence in ambient water ranges from 32 - 3000 species (Thacker & Freeman 2012). The bacteria taxa identified in sponges varied with every study and a complete list of symbiotic species has not been produced for any sponge species, as each project focused either on specific bacteria genera or identified taxa to the phylum level. Phylum Proteobacteria represents around 50% of the sequenced bacteria from sponges, but in total over 40 different phyla have been extracted from sponges so far, indicating the range and complexity of symbiosis (Webster & Taylor 2012; Pita *et al.* 2016).

Another issue with the identification of symbiotic bacteria in sponges is that the taxonomic range appears to change with bacteria supply in different water conditions (Pita *et al.* 2016). Changes in the community of symbiotic bacteria in sponges appear to be triggered by stress factors such as temperature and disease or with seasons; however, this is species specific and these observations may not be universally applicable, as for example, the symbiotic bacteria in *Halinclona* spp. were found to remain stable throughout the seasons (Hoppers *et al.* 2015; Pita *et al.* 2016). One study kept sponges in a sterile laboratory setting for six months so that symbiotic bacteria could not be obtained from the water, and only one symbiotic bacterium – *Pseudomonas* sp. was present in the sponges (Böhm *et al.* 2001). This would suggest that sponges obtain most of their symbionts from the ambient environment. Alternatively, it is also possible that laboratory conditions placed the sponges under such stress so that they expelled or consumed their symbionts. However, this represents a knowledge gap, as no experiments testing the effect of stress on sponge symbionts were found when searching the literature but the algae in *Lubomirshkia baicalensis* were expelled due to unidentified stress mechanisms (Kaluzhnaya & Itskovich 2015). This indicates the complexity of sponge symbiosis and the lack of knowledge surrounding this topic.

Table 2.2. Summary of studies focusing on symbiotic bacteria in sponges and their function.

| **Species and study aim** | **Main findings** | **Authors** |
|---|---|---|
| Infection of *Suberites domuncula* by natural microbes | • Sponges kept in the laboratory for 6 months had only 1 species of symbiotic bacteria – *Pseudomonas* sp.<br>• High numbers of bacteria kept in bacteriocytes | Böhm *et al.* (2001) |
| Bacteria associated with *E. fluviatilis* | • Six main phyla of bacteria: Actinobacteria, Bacteroidetes, Chlamydiae, Proteobacteria, Planctomycetes, and Verrucomicrobia | Costa *et al.* (2013) |
| Bacteria isolated from organisms including marine sponges | • Bacteria isolated included: *Bacillus* spp., *Rhodococcus* spp. and *Streptomyces* spp. | Eythorsdottir *et al.* (2016) |
| Bacteria found in *S. lacustris* | • Four main bacteria groups: Actinobacteria, Alphaproteobacteria, Betaproteobacteria and Chloroflexi | Gernert *et al.* (2005) |
| Bacteria community of *Haliclona* sp. and antimicrobial properties | • Sponge bacteria different from those in the water<br>• Bacteria isolated included: *Kistimonas* spp., *Serratia* spp., and *Candidatus* spp.<br>• Sponge (with symbionts) inhibited bacteria including *Staphylococcus aureus*, VRE, and *Vibrio parahaemolyticus* | Hoppers *et al.* (2015) |
| *Pseudomonas* spp. in *E. fluviatilis* | • 90 different *Pseudomonas* spp. isolates from sponges<br>• *Pseudomonas* spp. was not main bacterial symbiont in *E. fluviatilis* | Keller-Costa *et al.* (2014) |
| Bacteria associated with *Fasciospongia cavernosa* | • Bacterial symbionts including *Alteromonas* sp., *Micromonospora* sp., *Pseudomonas* sp., *Roseobacter* sp., *Saccharomonospora* sp., *Salinobacter* sp., *Streptomyces* sp., and *Vibrio* sp.<br>• These bacteria showed resistance to heavy metals and antibiotics | Selvin *et al.* (2009) |

Symbiotic bacteria have only recently been discovered in freshwater sponges (Gernert *et al.* 2005; Costa *et al.* 2013) but similarly to marine sponges, these freshwater organisms harbour a range of bacteria. The phyla of bacteria in freshwater sponges included Actinobacteria and Proteobacteria (Gernert *et al.* 2005; Costa *et al.* 2013). Even within a single bacterial genus there is wide variation as one study, solely focused on *Pseudomonas* spp., found 90 different strains (Keller-Costa *et al.* 2014). However, these represent a minor proportion in the taxonomic range of freshwater sponge symbionts. Unlike marine sponges, the symbiotic bacteria in freshwater sponges have not been found

throughout the freshwater sponge tissue but only occurred in vacuoles within the archaeocytes (Gernert *et al.* 2005). However, even with the restricted distribution of bacteria in freshwater sponges, these contain numerous symbionts including the phyla actinobacteria and Chloroflexi (Gernert *et al.* 2005; Costa *et al.* 2013). The importance of *Pseudomonas* to freshwater sponges as highlighted above also follows for marine sponge with *Aplysina fulva* harbouring 10 sponge-specific *Pseudomonas* which differed in 16S profile from those found in the water (Hardoim *et al.* 2009).

The bacteria isolated from sponges have also been found to reflect pollutants in the surrounding habitat. Bacteria associated with sponges in coastal bays with high influxes of heavy metals showed resistance to heavy metals including copper, lead, cadmium, and mercury (Selvin *et al.* 2009). In addition to resistance to heavy metals, many other sponge bacteria including *Pseudomonas* and *Bacillus* have also shown resistance to antibiotics including ampicillin, erythromycin, and tetracycline (Selvin *et al.* 2009; Hoppers *et al.* 2015), but it is unclear whether this resistance reflected antibiotic selection in the water or if it was a naturally occurring trait in these bacteria.

All of the above studies have the problem of differentiating between symbiotic bacteria and those being filtered by the sponge at the time of collection (Thacker & Freeman 2012). Some studies have compared sponge bacteria with those in the surrounding water, finding that bacteria in sponges often represented a smaller taxonomic diversity, but also contained some bacterial species not present in the water (Hoppers *et al.* 2015). The bacteria not found in the water were, therefore, assumed to be sponge symbionts. However, there was also evidence that sponges may obtain some of their symbionts from the environment (Thacker & Freeman 2012). There is experimental evidence for sponges' ability to concentrate their symbiotic bacteria from the water. Yet this has been reported to occur at up to 100 fold reduced rates compared to the concentration of 'food' bacteria (Wehrl *et al.* 2007). This further complicates the divide between 'food' bacteria and symbionts, because aquatic bacteria can be incorporated into the sponge tissue.

More information is needed on the symbiotic bacteria found in sponges as results and conclusions from previous research projects have varied greatly between individual studies and sponge species with 32-3000 species in each sponge sample (Thacker & Freeman 2012). With the rise in the availability of Next Generation DNA sequencing, it is becoming easier to produce a full list of symbionts, but the issue of separating symbiotic

species from environmental taxa being filtered by the sponge makes the interpretation of data difficult (Thacker & Freeman 2012). Pyrosequencing studies have confirmed high abundances of the same bacteria phyla previously recorded from traditional gene sequencing, however, some additional phyla, with a presence in low abundance have also been added (Webster & Taylor 2012). These advanced sequencing methods could be used for further exploration of differences in the symbiosis between bacteria and sponge species, particularly with varying water conditions and seasons. It is also necessary to test if bacteria are required for the survival of sponges and to further investigate the roles of symbiotic bacteria within sponges (Webster & Taylor 2012; McFall-Ngai *et al.* 2013).

## 2.7 Gene transfer between bacteria and its facilitation by host organisms

Conjugation is the most common method of HGT which can occur when bacteria come into close proximity forming direct cell-to-cell connections through which genetic information, e.g. a plasmid, can pass from a donor to recipient cell (Figure 2.4). This creates a transconjugant bacterium with genetic components from both parental (donor and recipient) bacteria (Massoudieh *et al.* 2007; Wilson *et al.* 2010). These genes can include antibiotic resistant traits which allow for the survival of the bacterium if it gets exposed to antibiotics (Dzidic & Bedekovic 2003). Due to the energy costs of replicating larger volumes of genetic material, genes acquired through conjugation can also be lost from bacteria, if the selective pressures, which made them advantageous, are removed (Dzidic & Bedekovic 2003).

Conjugal gene transfer of antibiotic resistance has been demonstrated with a range of bacteria *in vivo*. The methods generally employed filter, agar or broth mating individually or in combination (Ghosh *et al.* 2011; Conwell *et al.* 2017). For all the experiments, two isolates with different antibiotic resistance profiles were combined before being placed into broth or onto agar for mating. The parents can also be filtered and the filter plated on agar to allow for gene transfer (Ghosh *et al.* 2011). After incubation, the bacteria are removed and put onto agar containing two or more antibiotics to select for bacteria which have successfully transferred ARG.

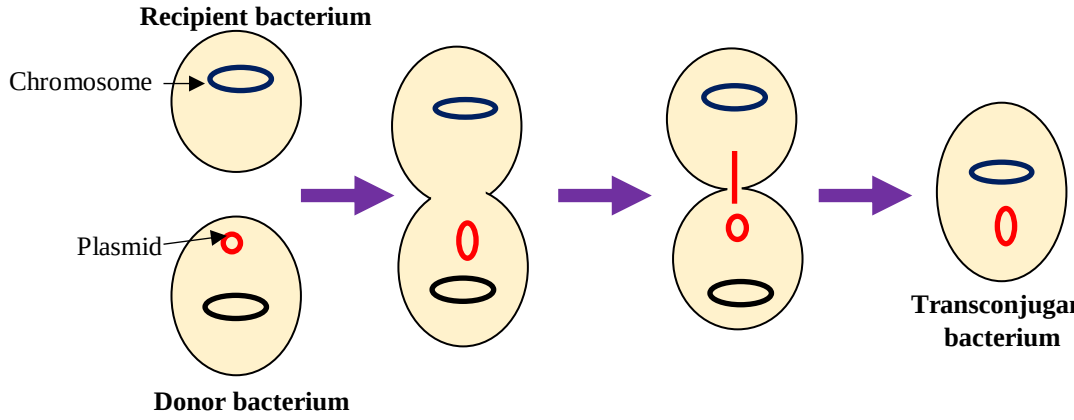



Figure 2.4. Plasmid conjugal gene transfer between Gram-positive bacteria.

Filter, broth and agar mating of bacteria have been used to demonstrate the potential of antibiotic resistance conjugal transfer from environmentally isolated bacteria. Studies have isolated ARB for conjugal transfer experiments from a range of sources including: fermenting sausages, house flies, and from the faeces of pigs, cats and dogs (Ghosh *et al.* 2011; Ghosh *et al.* 2012; Novais *et al.* 2013; Doud *et al.* 2014; Jahan & Holley 2016). In these studies, the antibiotic resistance profiles of isolates were established before some were selected for conjugal transfer experiments. These experiments all found that a minimum of two bacterial strains isolated from the organism tested were capable of conjugal gene transfer of antibiotic resistance. The transfer efficiency of these varied depending on the bacteria and the study. In the examined studies, the highest transfer efficiency (T/D 5.5 x $10^{-3}$) has so far been recorded between *E. faecalis* strains isolated from house flies and the lowest (T/D 3.3 x $10^{-8}$) for the interspecies transfer from *E. faecium* to *Listeria monocytogenes* isolated from fermenting sausages (Doud *et al.* 2014; Jahan & Holley 2016).

Conjugal transfer can be successful in a range of different conditions and surfaces and can take place in a range of different environments including seawater, freshwater, plants, and soil (Ashelford *et al.* 1997; Séveno *et al.* 2002). Conjugal transfer has also been recorded in biofilm and in model systems to imitate the human colon of an infant (Massoudieh *et al.* 2007; Haug *et al.* 2011). The erythromycin transfer from *E. faecalis* to *L. monocytogenes* was tested in a human colon model which contained faeces from infants to provide natural commensal bacteria (Haug *et al.* 2011). Results showed that resistance not only passed to the intended recipient but also to *Enterococcus avium*

present in the faeces, therefore indicating that conjugal transfer could be occurring in the human gut between newly introduced and pre-colonized bacteria.

There are many studies for *in vivo* conjugal transfer including those highlighted above, but comparatively few studies have been conducted within the natural environment (Bruun 2001). Conjugal transfer has been observed in the gastrointestinal tract of a number of different organisms including house flies, cockroaches, and mice (Lester *et al.* 2004; Akhtar *et al.* 2009; Anacarso *et al.* 2016). To test conjugation in organisms they were fed an appropriate food soaked in the donor or recipient bacteria. Once both bacteria were consumed, transconjugants were isolated from the organism or their faeces with transconjugant to donor ratios between $10^{-3}$ and $10^{-4}$ recorded (Table 2.3). It was also found that transconjugants persisted or were created in the gastrointestinal tract of cockroaches for up to 8 d after the last consumption of the parent bacteria (Anacarso *et al.* 2016). This further highlights the potential for organisms to create and retain ARB.

Table 2.3. Summary of selected conjugal gene transfer experiments inside organisms, including the bacteria tested and the transfer efficiency. (T/D = Transconjugant to donor ratio, cfu = colony forming units)

| **Species tested** | **Main findings** | **Authors** |
|---|---|---|
| Tetracycline resistance between *E. faecalis* in house flies | • Transconjugants were present in the flies after 24 h<br>• Maximal T/D: 1.4 x $10^{-3}$ cfu per fly | Akhtar *et al.* (2009) |
| Kanamycin resistance between *E. coli* in cockroaches | • Transconjugants present in faeces after 4 d and recorded from all cockroaches after 11 d<br>• Maximal T/D: $10^{-3}$ cfu $ml^{-1}$<br>• Transconjugants present in faeces for 8 days after feeding with parents ceased | Anacarso *et al.* (2016) |
| Kanamycin resistance between *E. coli* and *Salmonella enterica* in cockroaches | • Transconjugants present in faeces after 6 d and recovered from 63% of cockroaches<br>• Maximal T/D: $10^{-4}$ cfu $ml^{-1}$<br>• Transconjugants present in faeces of organisms where conjugation occurred for 8 days after feeding with parents ceased | Anacarso *et al.* (2016) |
| Erythromycin resistance between *E. faecium* in mice | • Transconjugants present in faeces after 24 h<br>• Maximal transconjugant number: $10^{6}$ cfu $g^{-1}$ faeces | Lester *et al.* (2004) |

The *in vitro* studies above showed that conjugal transfer of antibiotic resistance was possible when organisms were feeding. The transfer could also occur with symbiotic bacteria in addition to those ingested as food (Haug *et al.* 2011). Sponges have been found

to contain ARB (Selvin *et al.* 2009; Keller-Costa *et al.* 2014), but no studies investigating conjugal transfer between bacteria in sponges were found. However, Lupo *et al.* (2012) suggested the role of filterfeeders for HGT indicating a knowledge gap to be filled.

## 2.8 Antimicrobial properties of sponges

As highlighted earlier (section 2.3) antibiotics are not able to treat bacterial infections as effectively as they once did and, unless new antibiotics are found, human society faces a post-antibiotic era in which, once again, bacterial infections cannot be controlled (Berendonk *et al.* 2015; Kenny *et al.* 2015). To prevent this researchers are attempting to identify natural products with antimicrobial properties (Govinden-Soulange *et al.* 2014; Hoppers *et al.* 2015). Sponges are one group of organisms tested because they are assumed to contain molecules which inhibit bacterial growth, without which the sponge tissue would be overgrown by bacteria (Böhm *et al.* 2001). Alternatively, bacteria can contain antimicrobial molecules which inhibit the growth of other bacteria and so the symbiotic bacteria in sponges may also be a source of antimicrobial compounds (Saurav *et al.* 2016).

The methods commonly used to test the antimicrobial properties of sponges involved investigating the growth inhibition of sponge extract on selected bacteria strains. To do this, sponges were extracted in specific solvents (Table 2.4), the extracts dried and resuspended in the same or a different solvent at a desired concentration. These extracts were tested on bacteria using either agar diffusion methods (Eythorsdottir *et al.* 2016; Saurav *et al.* 2016) or by measuring the optical density of a bacterial broth culture in the presence of the test compound (Pejin *et al.* 2014). The agar diffusion method added sponge extract either to a well cut into the agar or onto a sterile filter disc where it subsequently diffused into the surrounding agar potentially preventing bacterial growth (Lawrence *et al.* 2009; Eythorsdottir *et al.* 2016). The optical density method involved the absorbance measurement of the bacteria - broth - extract suspension After allowing time for bacterial growth and comparison of the suspension's absorbance to a control, inhibition can be detected (Hoppers *et al.* 2015). The agar diffusion method has also been used to screen for extracts that inhibit bacterial growth before finding the minimal inhibitory concentration through measurements of optical density (Marinho *et al.* 2010; Govinden-Soulange *et al.* 2014; Hoppers *et al.* 2015).

Table 2.4. Antimicrobial properties of sponges, including the effects of sponge tissue and symbiotic bacteria (MIC = Minimal inhibitory concentration; MIZ = Minimal inhibitory zone)

| **Sponge extract** | **Main findings** | **Authors** |
|---|---|---|
| Aqueous extracts of marine species containing symbionts including sponges | • 10% of sponges showed antimicrobial properties<br>• Bacteria including Actinobacteria were responsible for the inhibitory effect.<br>• Least inhibited – *E. faecalis*<br>• Most inhibited - *Candida albicans* | Eythorsdottir *et al.* (2016) |
| *Biemna tubulosa* and *Stylissa* spp. dichloromethane and methanol extract. | • MIC range for *B. tubulosa* and *Stylissa* spp. (mg $ml^{-1}$):<br>*E. coli* - 2.18-2.55,<br>*E. faecalis* - 2.18-2.55<br>*S. aureus* - 4.36-5.09 | Govinden-Soulange *et al.* (2014) |
| *Haliclona* sp. extracted in methanol, dichloromethane, or dichloromethane with methanol. | • MIZ (mm): *E. coli* and *P. aeruginosa* - 1.5, *V. parahaemolyticus* and VRE - 2.0, *S. aureus* - 3.0, *Micrococcus luteus* - 7.0, *Bacillus subtilis* - 8.5<br>• MIC (mg $ml^{-1}$): *S. aureus* - 50, VRE - 25, *V. parahaemolyticu* - 10 | Hoppers *et al.* (2015) |
| Aqueous and ethanol extracts from 12 marine sponges | • In a test of 44 bacteria strains, only two sponges did not inhibit bacterial growth. *Petromica citrina* prevented the growth of 30 strains of bacteria<br>• Reference strains were more inhibited than clinical strains<br><br><u>Reference strains (n = 18)</u><br>• No inhibition by sponge extracts: *Acinetobacter calcoaceticus, Enterobacter cloacae, E. faecium,* and *P. aeruginosa*<br>• *M. luteus* and *S. aureus* were inhibited by the most sponges (8 and 7 respectively)<br><br><u>Clinical strains (n = 26)</u><br>• No inhibition by sponge extracts: *Citrobacter freundii, E. cloacae, E. faecalis, Klebsiella pneumoniae*, and *P. aeruginosa*<br>• *S. aureus* and *Enterococcus* spp. were inhibited by the largest number of sponges (6 and 5 respectively) | Marinho *et al.* (2010) |
| Methanol or acetone extracts of *Ochridaspongia rotunda* | • Inhibition of quorum sensing and biofilm production of *P. aeruginosa*<br>• Methanol extracts were more effective than the antibiotic streptomycin. Ampicillin was more effective than sponge extracts. | Pejin *et al.* (2014) |

| | | |
|---|---|---|
| 14 marine sponges extracted in chloroform, or chloroform with methanol | • Crude extracts from four sponge species inhibited the growth of *E. coli, P. aerugonsa* or *B. subtilis* and prevented quorum-sensing.<br>• Eight of the sponge species did not inhibit *E. coli, P. aerugonsa* or *B. subtilis* growth. | Saurav *et al.* (2016) |

Only a selected number of bacterial taxa have been tested for growth inhibition by sponge extracts. Among the most commonly tested bacteria were *E. coli, Enterococcus* spp., *P. aeruginosa* and *Staphylococcus aureus*, which are all opportunistic pathogens of humans and increasingly problematic due to rising antibiotic resistance in selected strains. The results of the studies varied, and some sponges were more effective against bacterial growth than others (Table 2.4). From the reviewed literature *Petromica citrina* was the sponge with the most effective antimicrobial properties, inhibiting the growth of 30 different bacteria strains from 17 species (Marinho *et al.* 2010). Several of the tested sponges including *Hymeniacidon heliophile* and *Oceanapia nodose* did not inhibit the growth of these bacterial species/strains. When 16 sponge species were tested against different bacteria including *Mycobacterium tuberculosis, E. coli* and *S. aureus*, bacteria were inhibited by 13 to 83% of sponge species (Marinho *et al.* 2010; Eythorsdottir *et al.* 2016).

Antibacterial effects of extracts were not only observed for marine but also for freshwater sponges as an extract from the freshwater species *Ochridaspongia rotunda* inhibited the growth of *P. aeruginosa* biofilm production by preventing the release of pyocyanin, a toxin produced by *Pseudomonas* which is used for cell communication or quorum sensing (Pejin *et al.* 2014). Quorum sensing relies on the release of signal molecules specific to each species which are detected by *lux* receptors on related bacteria (Skindersoe *et al.* 2008). If the numbers of these bacteria are sufficient, they can enter a virulence mode whereby they replicate, potentially causing disease if they are within a host organism (Skindersoe *et al.* 2008). Therefore, to control virulence, products offering anti-quorum sensing properties are being investigated. These studies were not limited to freshwater sponges as Skindersoa *et al.* (2008) found *Luffariella variabilis* contained molecules including manoalide which inhibited *lasB::gfp*(ASV) fusion in *P. aeruginosa.* This indicated that sponges can interfere with the cell-to-cell signalling and subsequent virulence exhibited by bacteria.

Inhibition of bacterial growth by specific fractions of sponge extracts has also been tested. Sponge extracts were fractionated by flash column or thin-layer chromatography to investigate further which chemical agents caused the antimicrobial effect (Govinden-Soulange *et al.* 2014; Hoppers *et al.* 2015). Individual fractions generally showed lower inhibitory effect than the crude extract (Hoppers *et al.* 2015), as each fraction had different properties, thus making a crude extract with a greater combined antimicrobial effect. However, the antimicrobial effect of *P. citrina*, which inhibited 30 of 44 tested bacteria, was from one molecule, halistanol-trisulphate (Marinho *et al.* 2012).

The bacterial symbionts located in sponge cells or tissues could also cause the antimicrobial properties recorded from sponge extract and so these have been tested against other bacteria after their separation from the sponge (Eythorsdottir *et al.* 2016; Saurav *et al.* 2016). The phyla of symbiotic bacteria found to exert the most pronounced antimicrobial effect were Actinobacteria or Proteobacteria (Eythorsdottir *et al.* 2016; Saurav *et al.* 2016). These were also found to be the most abundant symbionts in sponges (see section 2.6). Their presence in a sponge may be required to prevent their host from being overcome by other bacteria which could result in disease or death. This effect would be related to bacteria releasing compounds such as hydrogen cyanide that are toxic to other bacteria, a mechanism which resulted in the screening of bacterial toxins for antibiotic properties (Keller-Costa *et al.* 2013). *Pseudomonas* appears to have a high association with sponges, both freshwater and marine. Keller-Costa *et al.* (2013) isolated 90 fluorescent *Pseudomonas* strains from *E. fluviatilis* with 44 inhibiting bacterial growth and 32 inhibiting protozoa growth. This indicates that the symbiotic community within the sponges is likely to prevent the growth of other organisms and so the incorporation of selected bacteria into sponges may be advantageous especially as not all bacteria are pathogenic to sponges. Fu *et al.* (2013) found that sponges could be infected by *Vibrio* but were not infected by *E. coli*.

# 3. **The ability of sponges to filter bacteria out of the water**

This section describes a trial to monitor the filtration of waterborne bacteria by freshwater sponges. This is one of the key interactions between sponges and bacteria which needed to be understood before other experimental work was possible. Feeding also indicates that sponges are healthy. The trial was conducted with the faecal indicator bacterium, *E. coli*, which is often found in aquatic environments and exhibits antibiotic resistance. This means it is one of the common bacteria that freshwater sponges will naturally be in contact with. When feeding rates have been quantified, simple viable counts of bacteria on agar plates have often been used. However, this method showed high variance between replicates, even from the same suspension, so the efficiency of additional methods for the quantification of bacteria was trialled in this project. Fluorescently labelled *E. coli* were used to allow for measurement of fluorescence intensity and flow cytometry for comparison with standard plate counts. These methods were all used for the quantification of bacteria before and after exposure to sponges. The novel aspect of the trial was the use of the fluorescence spectrometer to monitor the removal of planktonic bacteria by sponges.

## 3.1 Introduction

Freshwater and transitional water bodies can receive high inputs of bacteria including *E. coli* from human and animal waste (Walk *et al.* 2007; Longo *et al.* 2010). This bacterial pollution can impact on the aquatic ecosystem and human health as sewage contains pathogenic bacteria and viruses (Cabral 2010; Longo *et al.* 2010). High inputs of faecal indicator bacteria i.e. exceeding an average of 200 cfu $ml^{-1}$ result in closure of bathing and drinking water sources (Kay *et al.* 2006). However, conventional sampling methods are culture-based thus taking a minimum of 24 h before bacterial pollution is detected (Ashbolt *et al.* 2001). To reduce human exposure to these conditions, methods which rapidly quantify bacteria are needed. Recently, the use of fluorescence-based methods, such as flow cytometry, have been applied to the detection of bacteria in water (Joachimsthal *et al.* 2004; Berney *et al.* 2007; Bigoni *et al.* 2014). Flow cytometry and qPCR are pre-established methods to rapidly quantifying bacteria in water (Berney *et al.* 2007; Noble *et al.* 2010), but there is scope for other methods to be developed.

In addition to being used for the detection of bacterial pollution in water, aquatic filterfeeders have the potential to remediate this pollution. Sponges are able to retain up to 90% of bacteria from ingested water, keeping them at concentrations that greatly exceed those in the ambient water (Reiswig 1975; Longo *et al.* 2010). There are estimates

that the sponges will then consume up to 83% of these retained bacteria, and so can contribute to water purification (Perea-Blázquez *et al.* 2013). Most sponge-based research has investigated the removal of bacteria by marine sponges, probably due to the larger size and longer lifespan of these filterfeeders. However, little is known about how seasonal freshwater sponges remove bacteria from the water. One species of bacteria likely to form part of the natural diet in freshwater sponges is *E. coli* due to its abundance in their freshwater habitat from WWTP and farm runoff (Flint 1987; Baudart *et al.* 2000; An *et al.* 2002; Ahmed *et al.* 2005). Specific strains of these bacteria also exhibit high levels of antibiotic resistance (Berendonk *et al.* 2015) and so sponge feeding could remove ARB from the environment where they could have passed resistance genes or cause infection. Therefore, this study has investigated the removal of bacteria from water by freshwater sponges with a focus on *E. coli.*

Several studies have quantified bacteria present in sponges and water by counts of colony-forming units on agar-based media (Reiswig 1975; Frost 1980; Milanese *et al.* 2003). These studies often had few replicates and their results showed high variability (Willenz *et al.* 1986; Milanese *et al.* 2003; Fu *et al.* 2006; Perea-Blázquez *et al.* 2013), which made it difficult to draw firm conclusions. Therefore, fluorescence-based methods such as flow cytometry could be used to quantify sponge feeding.

This study involved a laboratory trial where *S. lacustris* were fed *E. coli* tagged with a green fluorescent protein (GFP). The GFP is a fluorescently labelled plasmid based on a GFPmut3 which is expressed with a $P_{lac}$ promotor (ATCC 2014). The expression of this protein results in cell fluorescence which can be detected visually and by fluorescence intensity and flow cytometry methods. This allowed for the quantification the bacteria in the sponge microcosm based on fluorescence. To validate the use of these methods, they were compared to the standard agar plate counts. Ultimately, these methods were used to indicate the effect of sponge filtering on the quantity of bacteria and evaluate the use of some fluorescence based methods for quantification of aquatic bacteria.

*Aim and Objectives*

The aim of this chapter was to investigate the ability of freshwater sponges to remove bacteria from water.

There were two objectives:

1. Monitoring of *E. coli* abundance in water with and without sponges.
2. Detection of bacteria in water by plate counts, fluorescence intensity and flow cytometry.

## 3.2 Methods

During the trial bacteria in water were monitored over a range of time-periods using plate counts, fluorescence intensity and flow cytometry with or without sponges (Figure 3.1). Bacteria abundance in control tubes and sponge microcosms were measured. Turbidity was also measured to monitor changes to particles in the solutions.

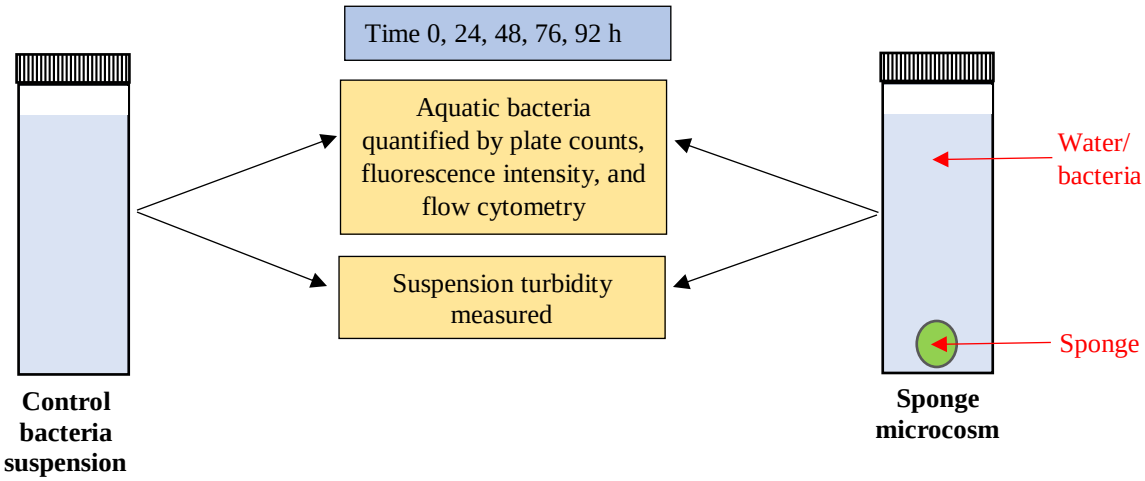



Figure 3.1. The measurements and experimental design used to quantify reduction in aquatic bacteria from sponges.

*Sponge sample identification*

For all experimental work on this project, the sponge species at the collection sites were identified through spicule preparation according to Cocchiglia *et al.* (2013). A 1 $cm^3$ sponge section was added to a boiling tube with 2 ml of 37% nitric acid. This was left in a fume hood for 16-24 h to dissolve the sponge tissue. The nitric acid was removed and replaced with 2 ml of water. This washing step was repeated twice leaving 10 min between each wash. Spicules were resuspended in ethanol and placed onto a dry slide. Canada basalm was added to permanently mount the slide (Ackers *et al.* 2007). Spicule identification of the species was completed at x 40 magnification on an Olympus BH-2 microscope according to Cocchiglia *et al.* (2013).

*Sponge collection from rivers*

Adult sponges (*S. lacustris*) were collected from Downhill River (Co. Londonderry) as 6 $mm^2$ discs using the top end of a 1 ml pipette tip. These were washed with autoclaved water (ELGA Purelab Ultra grade, 121 ºC, 20 min) and placed into 5 l of aerated mineral water for 24 h.

*Bacteria culture and experimental set up*

A fresh 24 h culture of *E. coli* GFP (ATCC ® 25922GFP™) was prepared in Tryptone Soya Broth (TSB, Oxoid) with 100 µg $ml^{-1}$ of ampicillin (Sigma-Aldrich – ampicillin sodium salt). The TSB was inoculated with bacteria from a stock culture stored at -80 ºC. This suspension was incubated at 37 ºC for 24 h before use.

Aliquots of 2 ml *E. coli* GFP suspension were added to universal tubes with 18 ml of UV treated mineral water (10 min at 254 nm). After initial measurements of fluorescence intensity, bacterial cell numbers, flow cytometry and turbidity, sponges were added to half of the tubes with 10 control tubes and 10 sponge microcosms in total (Figure 3.1). Fluorescence intensity, bacterial numbers, and turbidity were measured in samples taken after 24, 48, 72 and 96 h. Flow cytometry counts were performed at 72 and 96 h only. Before sampling, the sponges were carefully removed from each tube and placed into separate 6 cm petri dishes. This allowed for the suspension in the tubes to be homogenised before sampling. After taking samples, the sponges were returned to their tubes and incubation continued at 20 ºC.

*Bacteria counts of aquatic bacteria on agar plates*

For sampling, a 50 µl volume from each tube was removed and diluted in an Eppendorf tube with 450 µl of autoclaved water. Four further tenfold serial dilutions were carried out as above before six 20 µl drops were plated onto MacConkey No. 3 medium (Oxoid). The plates were incubated for 24 h at 37 ºC before counting. After the plate counts the bacterial number was calculated as N $ml^{-1}$.

The clearance rate of the sponges was calculated according to Fu *et al.* (2006) with the following formula:

$$CR = [\ln (C_0 - C_t)\ V/\ (WT)] \qquad \textbf{Equation 3.1}$$

where CR = clearance rate, $C_0$ = initial concentration, $C_t$ = final concentration, V = volume, W = sponge wet weight, and T = time.

*Fluorescence intensity quantification methods for bacteria in water*

A fluorescence spectrophotometer (Varian - Cary Eclipse) was used to measure fluorescence intensity. Preliminary calibration of signal peaks had shown that excitation at 501 nm and emission at 514 nm were optimal for the *E. coli* GFP. Mineral water was used as a blank before the fluorescence of each sample was measured.

*Flow cytometry counting methods for bacteria in water*

600 µl of each sample was added to a separate Eppendorf tube at 0 and 72 and 96 h into the trial. To each of these, 100 µl of Flow-count Fluorospheres (Beckman Coulter) were added. These were vortexed before 600 µl of each sample were loaded into a flow cell. The number of fluorescence cells was measured by flow cytometry (Beckman Coulter - gallios). The flow cytometer was calibrated to the signal for *E. coli* GFP, and *E. coli* TOP10 (Thermo scientific) without any fluorescence label was used as a negative control. For each sample 4,000,000 counts were carried out, to estimate the number of *E. coli* GFP cells per µl of sample. Counts were carried out in triplicates and converted to N $ml^{-1}$.

*Turbidity of bacterial suspension*

This parameter was monitored as a proxy for the small particulate matter in the suspension, i.e. individual live and dead cells, aggregates or cell fragments. Turbidity in water was quantified using light attenuation according to EN ISO 7027. While this international standard requires a wavelength of >860 nm, USEPA also describes turbidity measurement procedures for non-regulatory purposes with instruments which have a spectral peak response between 400 and 600 nm. In this study, turbidity was measured as light attenuation by measuring the absorbance of the sample with a spectrophotometer at 450 nm. The absorbance reading was correlated with a turbidity standard (Formazin, 400 NTU) to calculate the turbidity in FAU (Formazin Attenuation Units).

*Data visualisation and statistical analysis*

The arithmetic mean and standard error were calculated for the controls and sponge microcosms for each method. These were used to plot line graphs with the changes in the controls and sponge microcosms over the experiment duration. Results from controls replicates and sponge microcosms were tested for normality using the Kolmogorov–Smirnov test in SPSS (IBM V22) for each analysis method. Except for the flow cytometry results, data was not normally distributed. Two-way analysis of variance (ANOVA) with replication was used to test for differences ($p<0.05$) in the flow cytometry counts between

treatment (controls and sponges) and time (0, 24, 48, 72 and 96 h). For all other measures, the Scheirer-Ray-Hare test was used to test for significant differences ($p<0.05$) between treatment and time as described above.

## 3.3 Results

*Bacteria counts of aquatic bacteria on agar plates*

The bacteria in the water of the control and sponge microcosms were measured using bacteria counts on agar plates, fluorescence intensity and flow cytometry. The turbidity of the suspension was also measured. At the end of the trial the viable bacteria in the controls were higher than initial counts, but they were lower in the sponge microcosm (Figure 3.2). There was a significant difference in the bacteria counts between the controls and microcosm (SS/MS=7.9, *df*=1, p=0.028), however, the differences over time and the interactions between treatment and time were not significant. The average clearance rates for the sponges over the 96 h trial was 1.84 $\pm$ 0.22 ml $g^{-1}$ $h^{-1}$ with a maximum of 3.65 ml $g^{-1}$ $h^{-1}$ recorded with one sponge. During the trial, some white colonies were observed on the MacConkey agar from the sponge microcosm. These colonies showed fluorescence under UV light, but were not included in the counts as they could have been symbiotic bacteria released from the sponges.

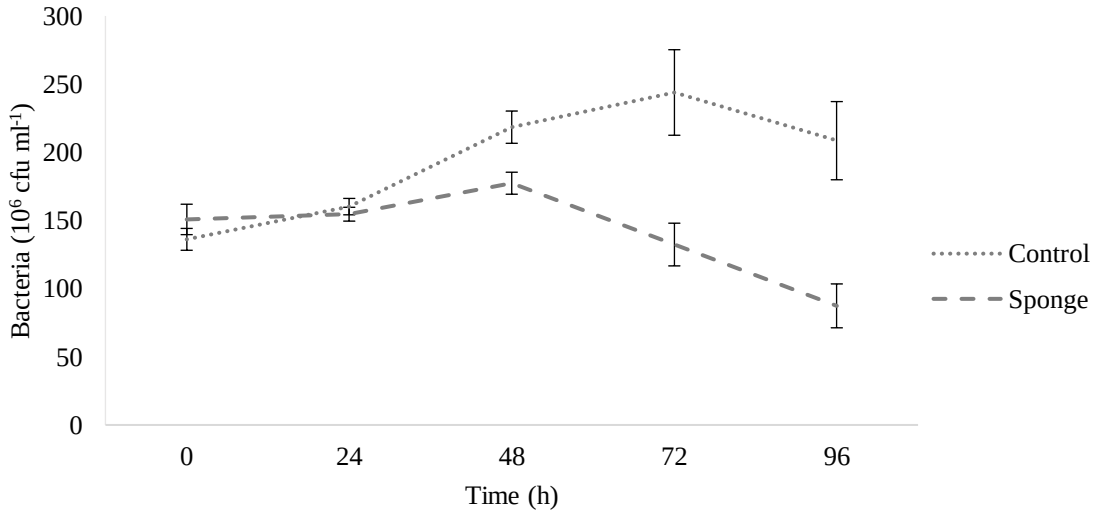



Figure 3.2. Arithmetic means of bacteria counts in water from sponge microcosms and controls over 96 h. Error bars are standard error of the mean.

*Fluorescence intensity of aquatic bacteria*

The fluorescence in the controls and sponge microcosms were low at the start but increased within a 24 h time-period remaining at a similar level thereafter (Figure 3.3). The fluorescence intensity was significantly different between the treatments and time (treatment- SS/MS=23, *df*=1, p<0.001; time- SS/MS=53, *df*=4, p<0.001). There was also a significant difference in the interaction between treatment and time (SS/MS=13, *df*=4, p=0.011), so the sponges and controls responded differently over time.

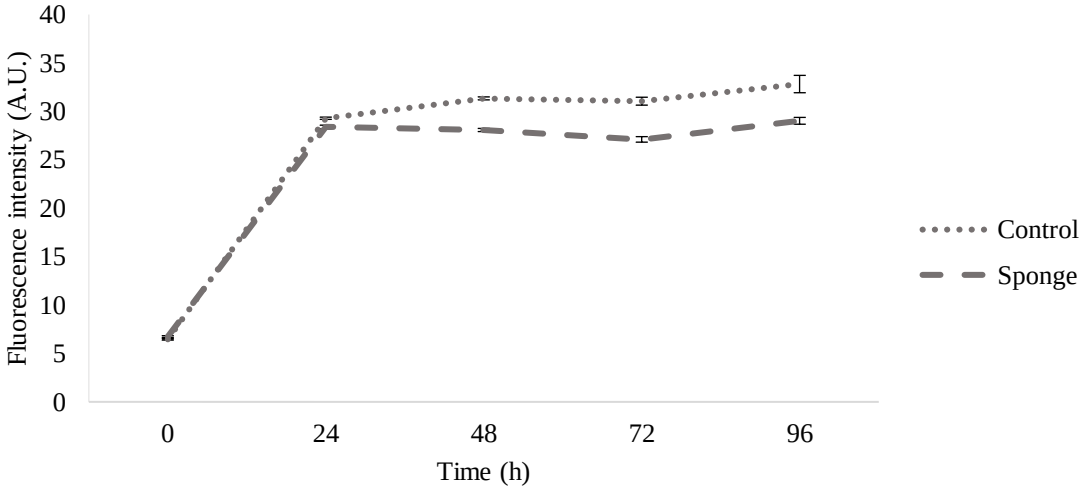



Figure 3.3. Arithmetic means of fluorescence intensity in water from sponge microcosms and controls over 96 h. Error bars are standard error of the mean.

*Flow cytometry count of aquatic bacteria*

The number of fluorescent cells did not show much variation both over time or between the controls and sponge microcosms (Figure 3.4). With overlapping error bars, the differences in the bacteria count in the controls and sponge microcosms could not be separated. There was no significant difference in the fluorescent cells present between treatment, time or the interaction between the two factors. The bacteria count from flow cytometry were 15 – 37 times higher per ml than on the agar plates so dead or viable but not culturable (VBNC) cells were counted.

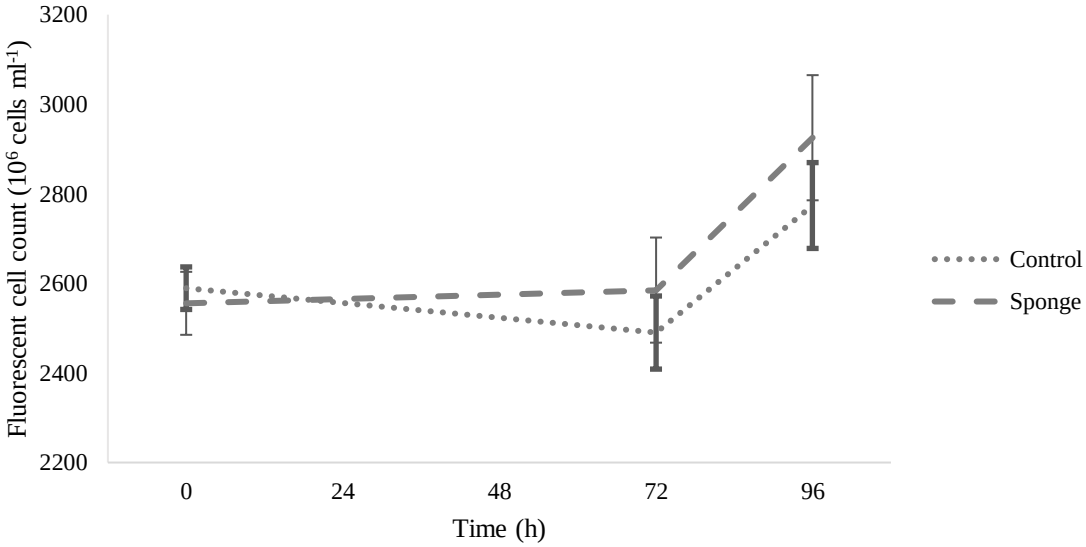



Figure 3.4. Arithmetic means of fluorescent cell counts in water from sponge microcosms and controls over 96 h. Error bars are standard error of the mean.

*Turbidity of bacterial suspension*

Turbidity readings in water from controls or sponge microcosms were similar at the start but higher in the microcosms thereafter (Figure 3.5). The turbidity was significantly different over time and treatment (treatment- SS/MS=7.9, *df*=1, p=0.005; treatment- SS/MS=83, *df*=4, p<0.001), but the interaction was not significant (SS/MS=5.3, *df*=4, p=0.255) so the response in both treatments was similar over time. The turbidity was expected to be higher with sponges due to feeding and the sponges themselves adding particles to the water.

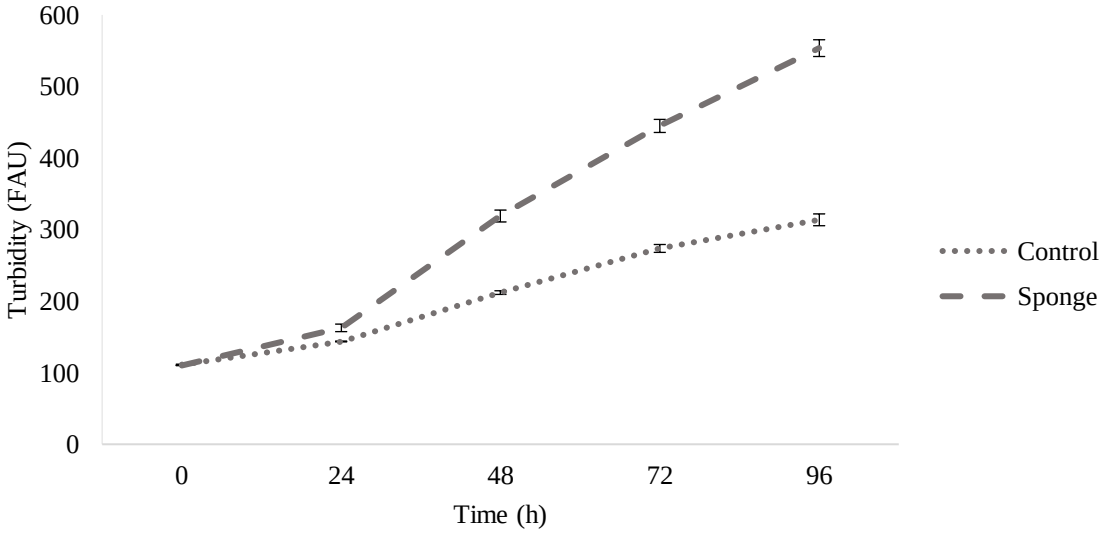



Figure 3.5. Arithmetic means of turbidity in water from sponge microcosms and controls over 96 h. Error bars are standard error of the mean.

Overall the water from sponge microcosms and controls had different fluorescence intensity, turbidity and agar plate bacterial counts. Fluorescence intensity and viable counts showed the same general patterns in the differences between the controls and sponge microcosms.

The main findings in this chapter were:

1. Sponges removed bacteria from the water as they filtered.
2. Plate counts were the best method for detecting changes in abundance of waterborne bacteria with sponge filtration. Fluorescence intensity can be used to monitor general changes in abundance, but flow cytometry requires further testing.

## 3.4 Discussion

This study showed the potential for filterfeeders to remove ARB from the aquatic ecosystem. Sponges fed on *E. coli* which were resistant to ampicillin. ARB have clinical significance because they can transfer ARG to other bacteria, or potentially infect people (Berendonk *et al.* 2015). Once ARB are in the water, they can enter the food web, for example, from irrigation water sprayed on crops (Solomon *et al.* 2002). Outbreaks of *E. coli* O157: H7 has significant health implications which have been linked to food products and their irrigation (Solomon *et al.* 2002; Mull & Hill 2009). Although wastewater treatment can effectively remove most of these bacteria, some are released and they are still found in surface waters (Mull & Hill 2009; Rana *et al.* 2011). Rana *et al.* (2011) found that WWTP were effective at removing 93% of the *E. coli* from water. However, WWTP are not the only source of bacteria as Ibekwe *et al.* (2011) found *E.* coli, coliforms, enterococci and total bacteria to be higher in an urban river than from two WWTP. Therefore, sponge filtration could offer the potential to remove these bacteria from the water.

Culture-based methods for the quantification of bacteria are slow and so more rapid methods of detection are being developed. These methods include enzyme based fluorescence and real-time PCR (qPCR) which have previously been used to detect bacteria including faecal indicator bacteria in water (George *et al.* 2000; Noble *et al.* 2010). The application of these methods for aquatic bacterial quantification are discussed below after an evaluation of each of the methods used in the current study.

*Bacteria counts of aquatic bacteria on agar plates*

Agar plating techniques for bacterial isolation and quantification has wide applications clinically and environmentally. It provides a standard method with worldwide use. The use of agar plates allowed for the detection of aquatic bacteria in this study. An overall decrease in bacteria numbers were recorded in the sponge microcosms compared to the controls, although there was high variability between replicates. The variability of the results in the viable bacteria was comparable with those from Reiswig (1975), Milanese *et al.* (2003) and Fu *et al.* (2006) who studied bacteria removal by marine sponges. Reiswig (1975), for example, found that the removal efficiency of *Haliclona permollis* could range from 30-90% even in healthy sponges. This has been attributed to the natural variability in filtering activity between individual sponges or as an indicator of poor sponge health (Reiswig 1975; Milanese *et al.* 2003). As all sponges were feeding in this

trial, they were considered healthy. Sponges do not only stop filtering when in poor health, they can also stop filtering at certain times in their lifecycle or when pollution is too high, thus reducing their removal of bacteria (Milanese *et al.* 2003). During flow conditions with high amounts of (inorganic) suspended sediment and near-bed transport of larger particles, sponges are unlikely to be feeding, as they contract when touched by objects (Elliott & Leys 2007). This would affect their ability to clear bacteria from the water and is also likely to apply to other filterfeeders.

At the start of the trial very high doses of *E. coli* GFP were added to the tubes ($1.13 \times 10^8$ – $1.69 \times 10^8$ cfu $ml^{-1}$). These were several orders of magnitude above the concentrations normally expected in aquatic systems, which in rural Irish streams ranged from $1.9 \times 10^3$ – $2.8 \times 10^4$ cfu per 100 ml (Daniels 2011). The initial loading of bacteria in this study was, however, comparable with other studies which had the goal of understanding sponge filtration. Similarly high *E. coli* concentrations of $1 \times 10^8$ to $2.20 \times 10^9$ cfu $ml^{-1}$ were added in these studies (Francis & Poirrier 1986; Willenz *et al.* 1986). Experimental evidence from marine sponges has shown that they can accumulate $7 \times 10^{10}$ of *E. coli* cells within each 1 $m^2$ per hour with a maximum clearance rate of 42 ml $g^{-1}$ $h^{-1}$ (Milanese *et al.* 2003; Fu *et al.* 2006), so the high bacterial loads were justifiable. In this study, the sponges would have retained a mean of $1 \times 10^8$ *E. coli* cells within each 1 $m^2$ of the sponge per hour, with a maximum clearance rate of 3.65 ml $g^{-1}$ $h^{-1}$. This indicated that the accumulation and clearance of *E. coli* were higher in marine sponges than freshwater sponge *S. lacustris*. However, in this study, there was a reduction in bacteria from $1.69 \times 10^8$ to $7.20 \times 10^7$, which was higher than values by Willenz *et al.* (1986), who recorded a reduction of *E. coli* numbers from $2.4 \times 10^7$ to $6 \times 10^7$ from the freshwater sponge *E. fluviatilis*. Therefore, the efficiency in sponge filtering varies with species and potentially with experimental design.

Sponges removed bacteria from the water though feeding, but even without this bacterial numbers in the water reduced over time. This was the result of the natural death of bacteria or from them sinking into the sediment (McFeters & Stuart 1972; Baudart & Lebaron 2010), and was exhibited by a slight reduction in the control bacteria. It has previously been demonstrated that 80% of *E. coli* remain viable in a laboratory setting after 5 d at 20 °C (McFeters & Stuart 1972), the same timeframe and temperature as used in this study and so only small reduction in viable bacteria should have occurred. However, if this

study was repeated in a river, bacteria lifespan would be shorter, as *E. coli* abundance in McFeters & Stuart (1972) study reduced to 0.2% in 5 d.

*Fluorescence intensity of aquatic bacteria*

This method does not only have application with fluorescently tagged bacteria as bacterial contamination can be detected from the natural fluorescence of bacteria cells (Dartnell *et al.* 2013). Measuring fluorescence intensity from fluorescence spectrometry provided a more stable method of quantification and highlighted that water in sponge microcosms had fewer bacteria than controls. However, this method detected a rise in fluorescence in controls and sponge microcosms over the first 24 h which was not related to a rise in plate counts. This meant general patterns could be monitored using fluorescence intensity, but that it cannot replace plate counts. The differences observed between the fluorescence intensity and plate counts were probably due to the quantification of fluorescence from dead or VBNC. The VBNC bacteria would have shown fluorescence, but they were not fit enough to reproduce on agar. These were probably exaggerated by fluorescence intensity, as Flint (1987) found *E. coli* survive in filtered water for 13 and >70 d at 25 ℃ and 15 ℃ respectively. Because the duration of the experiment was five days, natural decay was unlikely to occur, particularly with the death of natural microbes as potential competitors through the UV sterilization of the water before the trial (Garcia-Armisen & Servais 2007; Hijnen *et al.* 2006).

To further understand the effect of dead and VBNC bacteria, additional tests are required if this method is to be used to quantify aquatic bacteria. Firstly, it should be determined whether cell lysis removes fluorescence from dead bacteria. Autoclaving or ethanol treatment would denature cell membranes, as it kills *E. coli* (Simmon *et al.* 2004; Yoon *et al.* 2012; Huffer *et al.* 2011). Autoclaving is particularly effective as temperatures in excess of 100 ℃ have been found to denature DNA within a few minutes (Lindahl 1993). This should also destroy the GFP protein and remove background fluorescence. However, Sheridan *et al.* (1999) found that DNA was still detectable from PCR of dead cells which were treated with autoclaving and 50% ethanol. This means that dead cells may need to be stained to remove background fluorescence in future testing. Once the effect of dead-cell fluorescence is understood, fluorescence intensity can be trialled again as a more sensitive method for bacterial detection.

This is not the only study to use labelled *E. coli* when feeding sponges, Willenz *et al.* (1986) used radioactively labelled *E. coli* to test the bacteria retention by *E. fluviatilis*. Unlike this study, they investigated the bacteria inside the sponges finding that their abundance initially increased to 2 x $10^8$ cfu $ml^{-1}$ at 12 h before decreasing to 5 x $10^7$ cfu $ml^{-1}$ at 48 h. The decline in the radioactive *E. coli* retained was attributed to the digestion of bacterial particles (Willenz *et al.* 1986). This indicated that sponge feeding should have destroyed the bacteria thus preventing their detection. Their study did not use plate counting to quantify the bacteria and therefore had no independent method to confirm that the reduction in radioactivity with sponges was due to the digestion of the bacteria.

*Flow cytometry counts of aquatic bacteria*

Flow cytometry results, were highly variable and yielded no easily discernible patterns and therefore, did not represent reliable alternative to plate counts of bacteria in this study. Further method development is needed as dead bacteria appeared to be quantified. This was not known until after the trial as initial testing had shown a lower count of dead bacteria. This method has been used to investigate the different groups of prey utilised by marine sponges with flow cytometry (Topçu *et al.* 2010; Perea-Blázquez *et al.* 2013). Perea-Blázquez *et al.* (2013), for example, used this method to separate the bacteria and cyanobacteria species *Synechococcus* and *Prochlorococcus* available to the sponges for feeding, but they did not need to distinguish between live and dead organisms which affected this current study. Previously, the addition of propidium iodide has been used by researchers to distinguish between live and dead cells during flow cytometry with green fluorescently stained *E. coli* (Berney *et al.* 2007). The propidium iodide binds to the damaged cells quenching the fluorescence from these cells at the same wavelength as the live cells. This method worked for live and dead counts of *E. coli* in freshwater, seawater and drinking water (Joachimsthal *et al.* 2004; Berney *et al.* 2007; Bigoni *et al.* 2014). Therefore, it is possible that the addition of propidium iodide to the methods in this study would have allowed for the filtering effect of sponges to be better quantified as binding to dead cells would prevent the detection of the GFP protein.

Previously, fluorescence methods such as Fluorescent *In Situ* Hybridisation (FISH) were used to identify the presence of bacteria in water (Joachimsthal *et al.* 2004; Pavlekovic *et al.* 2009; Baudart & Lebaron 2010) and so this is already an established alternative to plate counts. With FISH, the fluorescent signal was emitted whenever the RNA sequence of the target bacteria was located. *E. coli* in freshwater and seawater has been identified

using FISH where a higher abundance of *E. coli* resulted in higher fluorescence intensity (Baudart & Lebaron 2010). However, the intensity of the fluorescence was always low due to the low abundance of the bacteria in the water with their highest recorded intensity of 0.51 with 2.44 MPN $ml^{-1}$ *E. coli*. Although Baudart & Lebaron (2010) found the intensity of fluorescence was related to bacterial abundance, they did not differentiate between live and dead *E. coli* in the water, so it is unclear whether this affected their method. The use of fluorescence methods can also be combined. Joachimsthal *et al.* (2004) measured the bacteria in water using both flow cytometry and FISH. Their use of specific bacterial oligonucleotides e.g. for *E. coli* in FISH allowed them to relate the bacterial counts from flow cytometry to the specific groups of bacteria recorded. The use of *E. coli* specific oligonucleotides with propidium iodide stained bacteria could allow for better quantification of bacteria from the water surrounding sponges in this study, and further use of this method with general aquatic bacteria.

*Turbidity of bacterial suspension*

The turbidity of the water was measured to detect particles. The turbidity in this study (both controls and sponge microcosms) increased over the trial. This was attributed to the growth of bacteria where turbidity rose as the number of bacteria increased (Dalgaard *et al.* 1994) and the creation of smaller particles for detection. It has been shown that turbidity increased with decreasing particle size where particles of 0.1 mm had turbidity readings of 0.1-2.0 NTU and particles of 1 mm had turbidity readings of 0.1 NTU when added at concentrations of 700 mg $l^{-1}$ (Sari *et al.* 2017) which was likely to occur as sponges filter bacteria breaking them into smaller particles. The higher turbidity in the sponge microcosms could also be due to the removal of sponges out of the tube for measurements which released sponge cells and fragments to the water, providing more particles that ultimately increased the turbidity. The turbidity in this trial was consistent between replicates, however, there can also be problems with this method through particle settling during readings and from aggregation of particles. Small particles such as bacteria and silt can show wide variations in turbidity readings which also varies with particle type (He & Nan 2012; Landers & Sturm 2013). It has also been suggested that turbidity was not an ideal measure for detecting bacteria in water (Madge & Jensen 2006), and so it was not used to estimate bacterial abundance in this study. Turbidity can be used to indicate pollution events e.g. floods when the suspended loads in the river increase, but it is unlikely to have application in the measurement of bacteria.

From this study, the best method for detecting changes in bacteria abundance was the widely applied plate counts. Reiswig (1975) attempted to replace agar counting with surface fouling and membrane filtration to quantify bacteria removal by sponges, but he also found plate counting was the best method. However, agar plates do not quantify all bacteria because injured organisms can lose viability (Flint 1987; Berney *et al.* 2007). Another problem with agar plates was the inability of some bacteria to grow on agar (Reiswig 1975). This was not a problem here as viable *E. coli* readily grow on MacConkey agar. The use of fluorescence to detect bacteria in water will provide a more rapid detection of *E. coli* (Hesari *et al.* 2016). Therefore, further tests with the fluorescence methods are recommended. A combination of results from two methods e.g. flow cytometry and FISH also allows for better determination of bacterial abundance (Joachimsthal *et al.* 2004).

There are other alternative methods to measure aquatic bacteria instead of plate counts, for example, enzyme based fluorescence where the addition of 4-methylumbelliferyl-β-D-glucuronid and subsequent hydrolysis to β-D-glucuronidase by *E. coli* resulted in fluorescence (George *et al.* 2000; Hesari *et al.* 2016). This method has been used to detect *E. coli* in water using a fluorescence spectrophotometer and identified *E. coli* within a few hours (George *et al.* 2000). Another rapid method used to quantify bacteria in water is qPCR. This has been used to quantify faecal indicator bacteria from freshwater, bathing water and wastewater (Lee *et al.* 2008; Varma *et al.* 2009; Noble *et al.* 2010). This method uses fluorescently labelled oligonucleotides which are specific to the target bacteria, releasing a signal proportional to the abundance to facilitate bacteria estimation (Fitzmaurice *et al.* 2004). However, the detection of *E. coli* with qPCR was also affected by dead cells unless stains to distinguish live and dead cells were used (Varma *et al.* 2009). Either of these established methods could also be trialled to detect the bacterial clearance rates of sponges and provide an alternative to culture-based techniques to quantify bacteria in water.

In addition to the results shown, it was found that adding *E. coli* GFP to sponges could be a useful test for filtration activity. Filtering sponges glowed when placed under UV light. When this exposure to UV light was limited to a few seconds, it did not seem to negatively affect the sponges. This study can also be used as a preliminary step to field trials to explore the potential applications of freshwater sponges for bioremediation of

bacteria pollution from the water whereby aquatic bacteria are reduced as sponges feed (Longo *et al.* 2010).

## 3.5 Summary

Sponges reduced the abundance of ampicillin resistant *E. coli* GFP from the surrounding water indicating that they feed on these ARB. Fluorescence intensity and viable bacteria counts can be used to monitor the reduction of bacteria in the sponge microcosms, but the use of fluorescence intensity cannot be used alone, as it is not easily converted to bacteria numbers and dead or inactive bacteria may still generate a fluorescence signal. This trial provided evidence for *S. lacustris* filtering bacteria which had application for all other trials in this project.

# 4. Biomonitoring of microbial water quality with freshwater sponges

This section contains information on the use of sponges to monitor microbial water quality using coliforms and enterococci. Laboratory trials tested the bacteria retention by sponges and whether the retention of bacteria reflected the time of exposure and the abundance of bacteria in the sponges' ambient environment. A subsequent field study investigated the bacteria retention in sponges across and along a river channel and how it related to their distance from a pollution source. The novelty of this section was the incorporation of different bacterial quantities and time of exposure to test bacterial retention by sponges. It was also unique in using sponges to identify point-source pollution in rivers.

## 4.1 Introduction

Pollution of freshwater systems can originate from both point and diffuse sources (O'Shea 2002). The pollution can be chemical or microbial. A primary source of microbial pollution is faecal matter from both human and animal sources, which may contain pathogens that pose a significant risk to human health (Baudart *et al.* 2000; Harwood *et al.* 2014). Sources of faecal contamination include ineffective WWTPs, agricultural runoff and ineffective septic tanks (Baudart *et al.* 2000; Ahmed *et al.* 2005). faecal indicator bacteria are monitored so that the public can be warned when the risk of water contamination is higher with an increased risk of exposure to harmful pathogens (Wiedenmann *et al.* 2006). By sampling drinking and bathing water for faecal indicator bacteria such as *E. coli* and *Enterococcus* spp. the risk of disease from human pathogens can be reduced (Ferguson *et al.* 2012; Wiedenmann *et al.* 2006). If bacterial counts exceed the acceptable threshold values set by regulatory agencies, the water should not be used for human consumption until bacterial counts drop to safe levels (Fu *et al.* 2006; Kay *et al.* 2006; Harwood *et al.* 2014).

Currently, there are several methods used for estimating the abundance of coliforms (in particular, *E. coli*) and *Enterococcus* spp. in water. These include non-culture methods such as qPCR and culture methods involving membrane filtration, selective media or defined substrates (Ashbolt *et al.* 2001; Noble *et al.* 2010). Conventional sampling for water chemistry which can also be applied for microbial parameters bases decisions on a single spot water sample taken at one point in time (Kirchner *et al.* 2004; Briciu-Burghina *et al.* 2014). Consequently, there is a need to develop time-integrating sampling techniques that can detect episodic microbial pollution. It is possible to detect changes in

water chemistry with regular sampling (Kirchner *et al.* 2004; Briciu-Burghina *et al.* 2014; Shore *et al.* 2017). This allows for the monitoring of episodic pollution, for example, phosphorus discharge from WWTP (Shore *et al.* 2017), but the application of these methods to microbiology is in its infancy.

Filterfeeders such as sponges process the bacteria within the water and potentially accumulate bacteria over a much longer timescale which would not be detected with a spot sample. When sponges are feeding they have the ability to select particles including bacteria which can be retained in their mesohyl (Wehrl *et al.* 2007; Topçu *et al.* 2010; Perea-Blázquez *et al.* 2013). A sponge sampled at any given point will contain bacteria which have been filtered from the water; most of these bacterial cells will be digested, but others will remain inside; along with the symbiotic bacteria of sponges (Fu *et al.* 2006; Wehrl *et al.* 2007). Therefore, the quantification of bacteria retained within sponges could be used as an indicator of the filter organisms' exposure to waterborne bacteria. The potential of freshwater sponges (*E. fluviatilis* and *S. lacustris*) to be used for biomonitoring bacteria within rivers was tested regarding their retention of coliforms and enterococci.

*Aim and objectives*

The overall aim of this section was to examine the ability of sponges to retain bacteria from the water thereby indicating microbial water quality. Enterococci and coliforms were used with sponges to see how the bacteria retained varied with time, abundance and location from pollution source. The following objectives were pursued with laboratory trials:

1. Investigation of the sponge's ability to retain *E. coli* and *E. faecalis*
2. Comparison of bacterial retention by sponges with different relative abundances of *E. coli* and *E. faecalis*
3. Comparison of bacterial retention by sponges with different exposure time to *E. coli* and *E. faecalis*

Field investigations aimed to achieve the subsequent objectives:

4. Investigation of the variability of bacterial abundance in sponges within and between sites, in consideration of their proximity to point source pollution.
5. Comparison of the concentration of coliforms and enterococci in sponge and water samples.

## 4.2 Methods

In total four trials were carried out (Figure 4.1). Two laboratory trials were carried out to further understand the retention of bacteria by sponges and how this related to the exposure. Trial 1 exposed sponges to both *E. coli* and *E. faecalis* at the same time, but at different abundance ratios. Trial 2 exposed sponges to either *E. coli* or *E. faecalis* before the sponges were removed and subsequent exposure to the other bacterial species, with the same concentration of each. These trials were conducted to establish if *E. coli* and *E. faecalis* retained by sponges reflected the abundance of bacteria which they had been exposed to in laboratory microcosms (trial 1) or the most recent exposure to bacteria (trial 2). Two field trials were then completed to investigate numbers of coliforms and enterococci in sponge populations of three different rivers (trial 3) and to assess if faecal indicator bacteria in sponges reflected the distance from a point source of faecal pollution (trial 4). The methods relevant to each trial are indicated below but the same general method shown in Figure 4.2 was used for all sections.

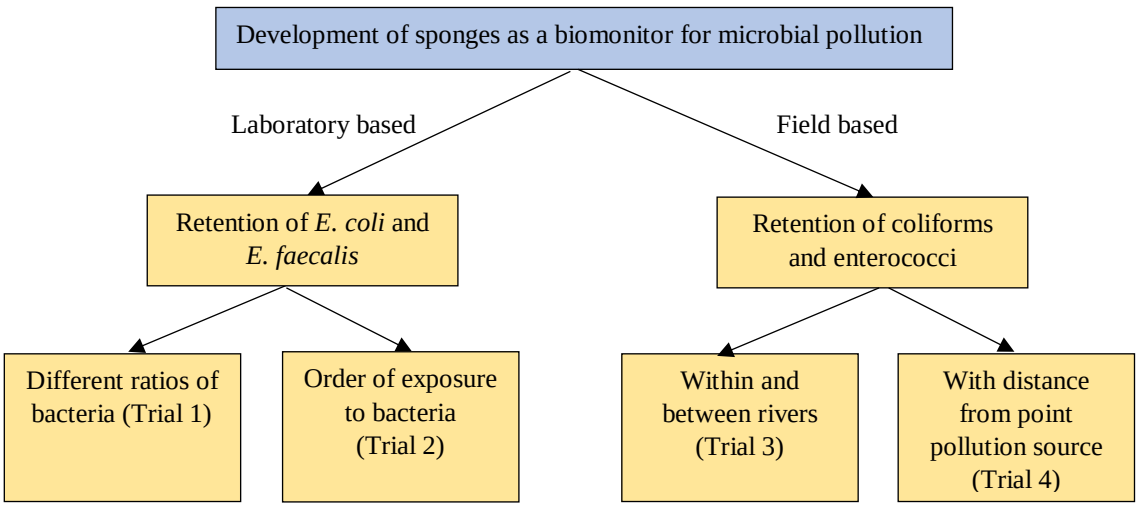



Figure 4.1. The investigation of sponges as biomonitors for microbial water pollution with four trials.

### *Sponge hatching (trial 1 and 2)*

Gemmules were treated using a modified method from Rasmont (1970) with submersion in 1% $H_2O_2$ for 10 min and storage in sterile water at 4 ºC until needed. Gemmules were hatched onto a piece of 4 $cm^2$ transparency film (Xerox type 1) in a 6 cm petri dish containing 10 ml UV treated (10 min at 254 nm) mineral water. The horizontal dimensions of each 7-day-old sponges was measured ($mm^2$) before they were used in trials.

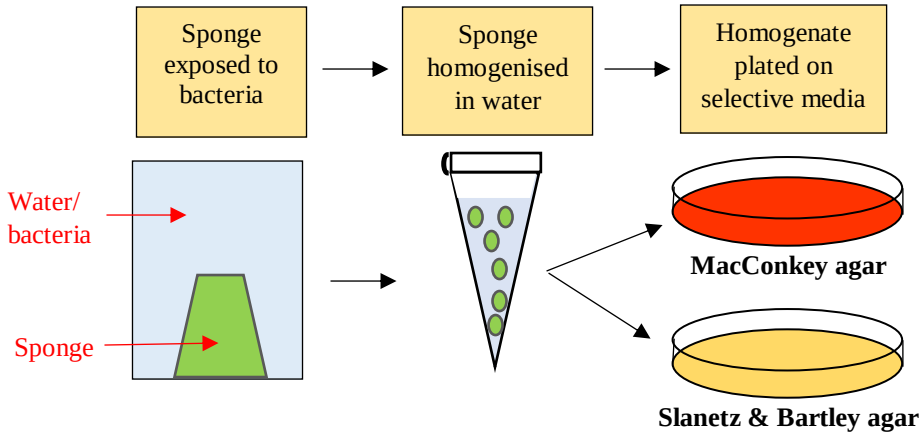



Figure 4.2. Experimental protocol to test sponges for retention of coliforms/ *E. coli* (MacConkey agar) and *Enterococcus* spp. (Slanetz & Bartley agar).

*Bacterial addition – (trial 1) ratios of E. coli and E. faecalis*

Universal tubes with 18 ml of UV treated mineral water had 2 ml of bacterial suspension added. The bacteria suspension contained different ratios of *E. coli* GFP (ATCC 25922GFP) to *E. faecalis* (MW01105 - Conwell *et al.* 2017). The trial was carried out in three runs but these were not combined as there was no significant difference between the initial bacteria concentration at the start of the experiments. There were 22 replicates for each of the following *E. coli*: *E. faecalis* ratios: 10:90; 50:50; 90:10. Sponges on transparency films were placed into the tube, so that they were leaning on the wall, to minimise settling of bacteria on sponge surfaces due to deposition. These tubes were kept at 20 ºC for 24 h.

*Bacterial addition – (trial 2) separate exposure to E. coli and E. faecalis*

Universal tubes with 19 ml of UV treated water had 1 ml bacteria suspension (either *E. coli* or *E. faecalis*) added before the sponge was introduced on transparency film as in trial 1. There were 16 replicates. Half of the tubes received the suspended *E. coli* GFP, the other half received the suspended *E. faecalis*. The tubes were incubated at 20 ºC for 24 h. After 24 h, sheets with sponges were removed and washed before they were placed in a fresh tube with 1 ml of the bacterial strain that the sponge had not been previously exposed to. These were incubated at 20 ºC for a further 24 h.

*Sample processing (trial 1 and 2)*

After 24 h the transparency film with sponges were removed and washed with autoclaved water (ELGA Purelab Ultra grade, 121 ºC, 20 min). The sheets of transparency film were then placed in a fresh tube with 20 ml of mineral water. These were left for a further 24

h. To end the trial, sponges/ sheets were once again washed. The sponges were scraped off the sheets and placed into an Eppendorf tube with 1 ml of autoclaved water. The Eppendorf tube was vortexed for 2 min to extract the bacteria before tenfold serial dilutions. The samples for bacteria analysis were tested for *E. coli* and *E. faecalis* using MacConkey No. 3 and Slanetz & Bartley agar respectively. The dilutions were plated onto each selective medium in six 20 µl aliquots. The plates were incubated at 37 °C for 48 h before colony counts.

*Sponge collection (trial 3)*

Sponges were collected from Orritor River (Co. Tyrone), Cavan River (Co. Cavan) and Rag River (Co. Cavan). Between rivers sponge communities differed in species assemblage and abundance (Table 4.1). Ten to twelve samples were collected from each river across a transect where sponges were in abundance. On collection sponges were cut into circular discs 6 mm in diameter using the top of a pipette tip and placed into a container with the stream water. Fragments of sponge were also collected for species identification as described in Section 3.2. All samples were transported back in an ice box.

Table 4.1. Sponge cover and species present in selected rivers with Irish Grid reference.

| **River** | **Sponge coverage** | **Species present** |
|---|---|---|
| **Orritor River** (479700, 277500) | Patchy growth on boulders and bedrock. All encrusting growth. Sponges white or green in colour. | *E. fluviatilis;* *S. lacustris* |
| **Cavan River** (369000, 241400) | Widespread growth on boulders, bedrock and bridges. All encrusting growth forms. Green where exposed to sunlight, white if shaded. | *E. fluviatilis* |
| **Rag River** (330300, 217900) | Widespread growth on boulders, vegetation, mussel shells, soft silty bed. Mainly encrusting growth form, but branched structure of *S. lacustris* on soft sediment. Sponges white or green. | *E. fluviatilis;* *E. muelleri;* *S. lacustris* |

*Sponge collection (trial 4)*

The recorded location of sponges in Cavan River appeared to be confined to a one kilometre stretch of the river (Figure 4.3). The sponge recorded furthest upstream was 50 m above the effluent for Cavan Glan Aqua WWTP and the last sponge in a downstream direction was found 1000 m downstream of this point. Sponges were sampled across four transects; 35 m upstream of the WWTP and three transects 100, 300 and 900 m downstream of the treatment plant. Upstream of the WWTP sponges were less abundant

so only 6 sponge samples were collected for bacterial analysis, but 20 samples were collected from the other sites. Depth and distance from the left bank were recorded for each sponge sample. Each sponge was collected as discussed above.

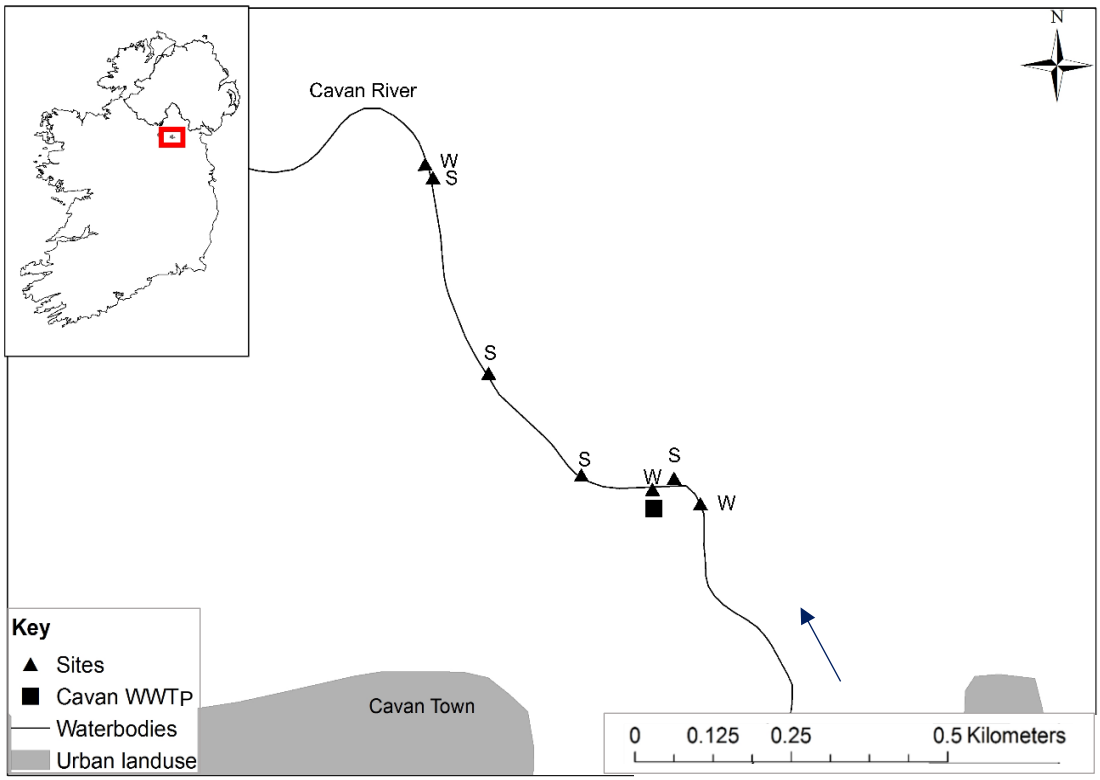



Figure 4.3. Locations sampled in Cavan River (trial 4) where S = sponge, W = water, → = direction of flow.

*Processing of sponge samples (trial 3 and 4)*

Sponge samples were washed with autoclaved water and pressed onto a paper towel for partial dryness. Sponge samples were weighed before being placed into an Eppendorf tube for cutting and plating. Each of the samples was tested for coliforms and *Enterococcus spp.* using MacConkey and Slanetz & Bartley agars respectively. The sponge discs were cut into fine pieces with sterile blades in 6 cm petri dishes and placed into an Eppendorf tube with 1 ml of autoclaved water. The samples were vortexed for two minutes to extract bacteria. A 10-fold serial dilution was prepared from this suspension. Aliquots of these dilutions were plated onto selective media in six 20 µl dots. The plates were incubated at 37 °C for 48 h before colony counts.

*Processing of water samples (trial 4)*

Water was sampled at three sites: 50 m upstream of the WWTP (15 m above first sponge), the WWTP effluent and 910 m downstream of the WWTP (5 m downstream of the last sponge sampled; Figure 4.3). For 24 hours, water samples were collected every half hour.

The Cavan River samples were collected with ISCO 6700 portable water samplers with 24 sterile sample bags. Two 250 ml samples were collected into each bag, one every 30 min. At the WWTP, 100 ml of the water sample was collected every half hour into a sterile bag using a composite water sampler (Hach Bühler 3010). The following data was also recorded for the effluent water in one-minute intervals: temperature, pH, DO (dissolved oxygen), COD (chemical oxygen demand), ammonia, phosphorus and suspended solids. Water quality data was summarised through the calculation of arithmetic 24 h means and standard error values.

The WWTP was a newly built facility (June 2015) commissioned to replace its predecessor, which had regularly failed to comply with regulatory effluent quality standards. The new WWTP is equipped with aerobic and anaerobic mixers, and clarifiers (Mr B. Mackow 2016, pers. comm 8$^{th}$ September) for the removal of sediments and nutrients, but it lacks a specific treatment for free-floating bacteria.

Upon collection, water samples were transported to the laboratory for further analysis. A 24 h composite sample from the two ISCO probes was prepared. Each bag was gently shaken before 100 ml was removed and placed in a sterile bottle. These bottles provided the undiluted samples. From each of the three undiluted samples, tenfold serial dilutions until $10^{-2}$ were prepared with autoclaved water (ELGA Purelab Ultra grade, 121 ºC, 20 min).

Bacteria from each dilution was estimated by filtering 100 ml of each water sample through sterile 0.45 µm filter paper (Sartorius stedim biotech) on a Millipore Microfil filtration system. The filter membrane from each water sample and dilution were used as follows: three replicates were plated onto MacConkey agar and three replicates were plated onto Slanetz & Bartley media to test for coliforms and *Enterococcus* respectively. The agar plates were incubated at 37 ºC before counting at 24 and 48 h.

*Data visualisation and analysis (trial 1-3)*

Counts were converted to cfu per mm$^2$ of sponge surface for each individual sponge as follows:

$$B_S = \frac{N}{\overline{A}} \qquad \textbf{Equation 4.1}$$

Where $B_S$ = bacteria in sponge, N = bacteria number per 1 ml, A = sponge area

The bacteria in all sponge replicates was used to calculate the arithmetic means and standard error values for each experimental group or site, for *E. coli/* coliform or *E. faecalis/* enterococci. For trial 2 and 3, this information was used to create bar charts showing the bacteria abundance in each experimental group/site. In trial 1 the *E. coli* and *E. faecalis* retained by each sponge was divided by the total bacteria added to the tube and converted into a percentage for *E. coli* and *E. faecalis*. The mean and standard error was calculated and plotted on a bar chart.

Statistical tests were carried out in SPSS v22. For trial 1 - 3, Kolmogorov –Smirnov tests showed that bacteria retention was not normally distributed. For each trial, the different treatments/sites were tested for significant differences ($p<0.05$) with Kruskal Wallis tests. Post hoc tests were pairwise comparisons with Bonferroni corrected Mann–Whitney U tests. This was used to test the following: difference in bacteria retention with the ratio *E. coli/ E. feacalis* (trial 1), differences in retention with order of exposure (first or second) and bacteria (trial 2), and differences between rivers (trial 3).

*Data visualisation and statistical analysis (trial 4)*

The bacteria cfu per $mm^2$ of sponge surface were calculated as above and the bacteria in the water per ml. Arithmetic means and standard error values of bacteria numbers in water or sponge were calculated for each river site and used for graphs. Bacteria numbers were tested for normality using the Kolmogorov – Smirnov test. Data was normally distributed. Therefore, one-way ANOVA with Least Significance Difference (LSD) post hoc testing was completed. The same tests were repeated with the bacteria per ml of water.

## 4.3 Results

The amount of *E. coli* and *E. faecalis* retained by sponges were quantified in a laboratory setting before the retention of coliforms and enterococci retention in sponges was investigated in rivers. Pilot investigations found freshwater sponges retained both *E. coli* and *E. faecalis*, which had not been present in gemmule-grown sponges until they were exposed to these bacteria. Bacterial concentration in sponges exceeded those in the water. Dead sponges also retained bacteria, but at a significantly lower concentration for *E. coli* ($U=86$, $p=0.019$). These results suggested that the bacteria on the dead sponges had settled on the surface, while the number of bacteria recovered from live sponges represented bacteria from the surface and those bacteria cells retained inside the sponges from water filtration. When sponges were collected from rivers and maintained in a laboratory setting to monitor their content of coliforms and enterococci, significant increases were observed for coliforms after 7 d and enterococci after 3 d. Therefore, bacteria from samples were quantified within 12 h of collection.

*Retention of E. coli and E. faecalis in sponges exposed to different ratios of both bacteria (trial 1)*

The percentage of bacterial retention by sponges was very low regardless of the initial bacterial concentration, and the bacterial retention of sponges never exceeded 0.5%. Retention values for *E. coli* were always higher than for *E. faecalis* (Figure 4.4). A significantly lower bacteria retention percentage was recorded for both *E. coli* and *E. faecalis* when these constituted 90% of the added bacteria suspension (*E. coli*- $U=95$ $p<0.001$; *E. faecalis*- $U=405$ $p=0.001$). *E. coli* percentage retained by sponges was significantly higher when it constituted 10 and 50% of the bacterial suspension (10%- $U=60$ $p=0.021$; 50%- $U=8$ $p=0.006$).

*Retention of E. coli and E. faecalis in sponges exposed to each bacteria species separately (trial 2)*

The sponges exposed in the sequence of *E. coli- E. faecalis* and *E. faecalis- E. coli* showed a higher retention of *E. coli* (Figure 4.5) but this difference was not significant for either exposure sequence ($U=23$, $p=0.902$). *E. coli* was retained in significantly higher abundance ($U=11$, $p=0.040$) when sponges were exposed to this bacteria type first (sequence *E. coli- E. faecalis*). However, regardless of the sequence of exposure to *E. faecalis* there was no significant difference in their abundance in sponges. Overall, the sponges retained bacteria from a longer time-period and not only the most recent 24 h.

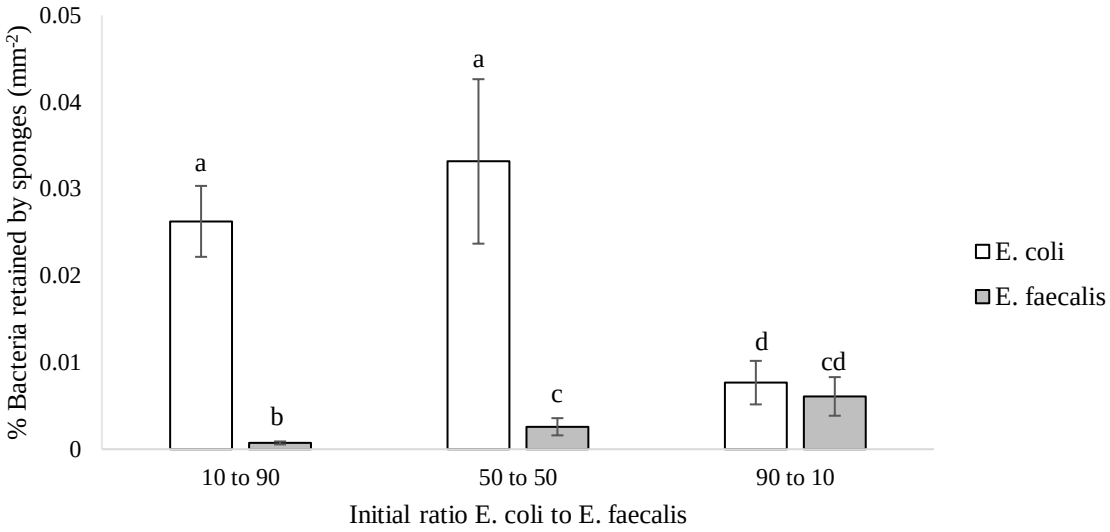



Figure 4.4. Arithmetic means and standard error values for the retention percentage of *E. coli* and *E. faecalis* by sponges in treatments with different *E. coli*: *E. faecalis* ratios. Different letters indicate significant differences ($p<0.005$) between bars.

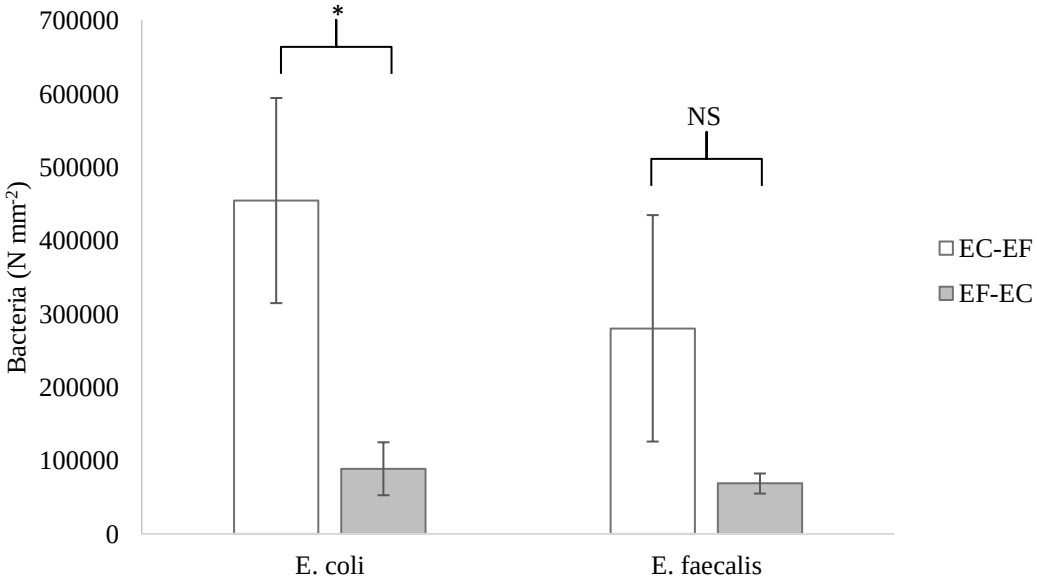



Figure 4.5. Arithmetic means and standard error values for counts of bacteria retained in sponges in treatments with sequential exposure to bacteria (EC-EF = first exposure to *E. coli*, then to *E. faecalis*; EF-EC = first exposure to *E. faecalis*, then to *E. coli*). * - significant ($p<0.05$), NS - not significant.

### *Coliforms and Enterococci retained in sponges from across individual river channels, and between different rivers (Trial 3)*

Within individual rivers, the variance of coliforms in sponges was not significant between transects. However, between rivers, there was greater variance in coliform numbers in the sponge (Figure 4.6a). The highest coliform numbers were recorded in sponges from Orritor River and the lowest from Rag River. There was a significant difference between

rivers (H=8.9, p=0.012). In pairwise comparisons sponge samples from Orritor River and Cavan River contained significantly higher coliform numbers than Rag River (U=32, p=0.020 and U=19, p=0.006 respectively). Bacteria in the river water was lower than in sponges (Appendix 3).

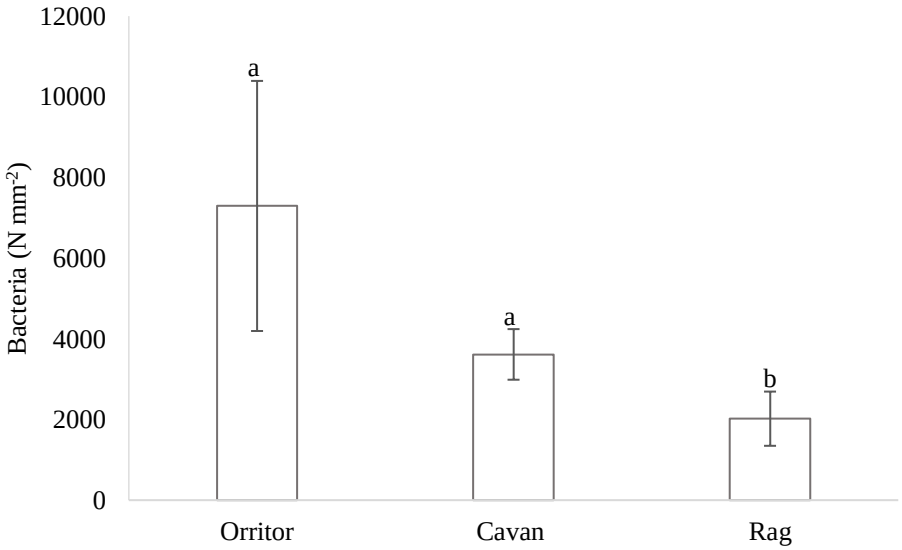



Figure 4.6a. Arithmetic means and standard error values for coliform retention in sponges from different rivers. Different letters indicate significant differences ($p<0.005$) between bars.

The numbers of enterococci in sponges were lower than those of coliforms. Similar to coliforms, the variance of enterococci in sponge samples was insignificant between transects within individual rivers. However, there was greater variance between rivers (Figure 4.6b). As for coliforms enterococci numbers were highest in samples from Orritor River and lowest in samples from Rag River. There was a significant difference between rivers (H=20, $p<0.001$). In pairwise comparisons, the Orritor River and Cavan River had significantly higher enterococci numbers than Rag River (U=4.5-6, $p<0.001$).

*Coliforms and Enterococci retained in sponges at different distances from point source of faecal pollution (Trial 4)*

Coliform abundance in sponge samples increased in the downstream direction of the Cavan River (Figure 4.7a). It was lowest upstream of the WWTP and very high at the site furthest downstream of the WWTP. Between locations the differences in variance of coliform abundance in sponges were significant (F=7.9, *df*=3, $p<0.001$). LSD showed that the coliforms in sponges 900 m downstream of the WWTP were significantly higher ($p\leq0.001$) than those in sponges from other locations. Bacteria in the river water was lower than in sponges (Appendix 3).

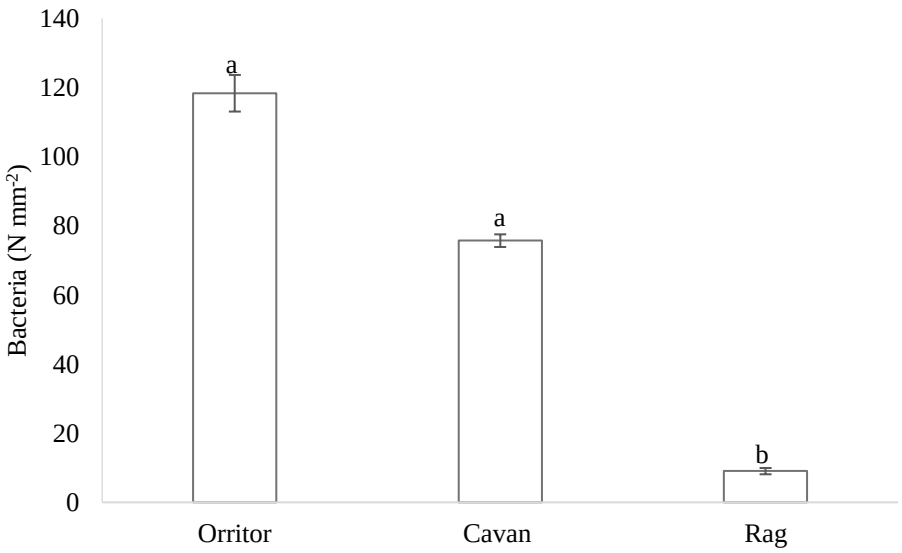



Figure 4.6b. Arithmetic means and standard error values for enterococci retention in sponges from different rivers. Different letters indicate significant differences ($p<0.005$) between bars.

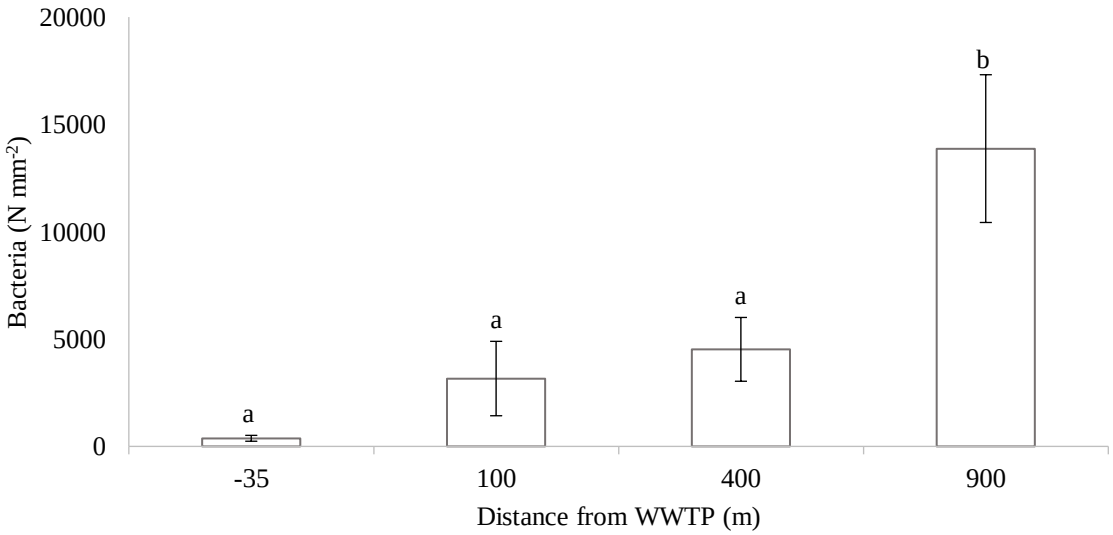



Figure 4.7a. Arithmetic mean and standard error values of coliforms in sponge samples with different proximity to the WWTP effluent discharge point. Site –35 m was upstream of discharge point. Different letters represent a significant difference in the results ($p<0.05$).

The enterococci abundance in the sponges decreased in the downstream direction (Figure 4.7b). Counts were highest in sponges located upstream of the WWTP (from unknown sources of pollution) and were lower downstream. Between locations, there was a significant difference in enterococci abundance ($F=4.6$, $df=3$, $p=0.006$). LSD showed that the enterococci in sponges above the WWTP were significantly higher than the bacteria in the sponges from all downstream locations ($p\leq0.002$). The enterococci abundance in the sponges was also significantly ($p<0.001$) lower than the abundance of coliforms in sponges.

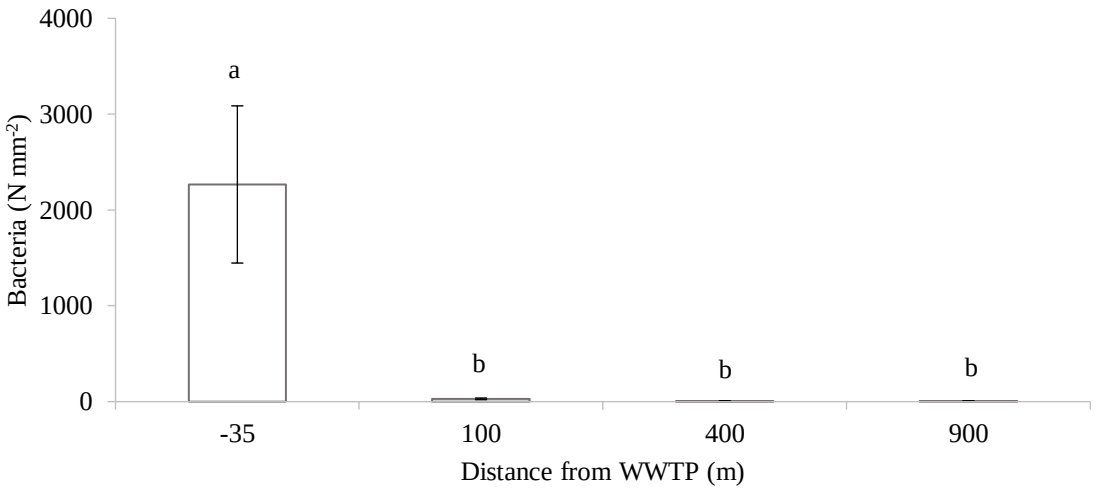



Figure 4.7b. Arithmetic mean and standard error values of enterococci in sponge samples with different proximity to the WWTP effluent discharge point. Site –35 m was upstream of discharge point. Different letters represent a significant difference in the results ($p<0.05$).

*Water samples with different distances from point source of faecal pollution (Trial 4)*

There were hardly any fluctuations in measurements of water quality parameters in the WWTP effluent over the 24 h period (Table 4.2). The temperature was 18 ºC, which was 4–5 ºC higher than in the main channel. The pH remained around 6.9. The DO was high (89%) and the COD low in comparison to a typical environmental limit value of 125 mg $l^{-1}$. The ammonia nitrogen ($NH_3$-N), phosphorus and suspended solids in the water were also very low. These showed that the WWTP was effective at removing both solid material and nutrients.

Table 4.2. Measurements of the WWTP effluent during the 24 h sampling period. Where DO = dissolved oxygen, COD = chemical oxygen demand

| | Arithmetic mean ± standard error |
|---|---|
| Temperature (ºC) | 18.41 ± 0.00 |
| DO (mg $l^{-1}$) | 8.42 ± 0.00 |
| pH | 6.91 ± 0.00 |
| Ammonia (mg $l^{-1}$) | 0.04 ± 0.00 |
| Phosphorus (mg $l^{-1}$) | 0.07 ± 0.00 |
| Suspended solids (mg $l^{-1}$) | 2.38 ± 0.00 |
| COD (mg $l^{-1}$) | 20.89 ± 0.01 |

The number of coliforms present within the water from Cavan over a 24 h period was highest upstream of the WWTP and lower in the wastewater effluent and downstream of

the last sponge (Figure 4.8a). Upstream of the WWTP the bacteria colonies were too numerous to count, however based on the growth at the different dilutions the coliforms were at least 10 times higher than at the other two sites. The coliforms numbers in the downstream river were slightly higher than in the wastewater but the difference was not significant (F=0.04, *df*=1, p=0.852). Nevertheless, the WWTP was not the main source of coliforms.

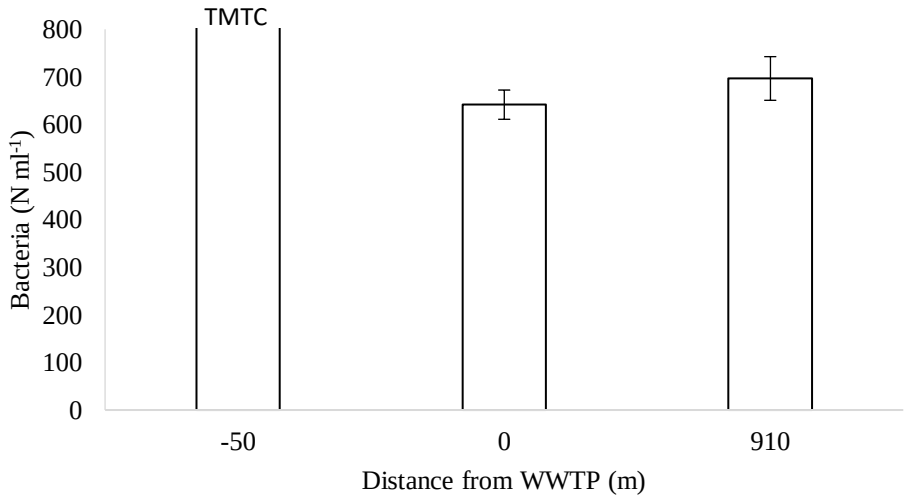



Figure 4.8a. Arithmetic mean and standard error values of coliforms in water samples with different proximity to the WWTP effluent discharge point. Site –50 m was upstream of discharge point. TMTC – too many to count. No significant differences in bacteria counts.

The abundance of enterococci in the water from Cavan over a 24 h sample was higher in the wastewater than in the main river (Figure 4.8b). Upstream of the WWTP had a lower abundance of *Enterococcus* spp. than downstream of the sponges but this was not significant (F=3.0, *df*=2, p=0.123). In river water, the numbers of enterococci were significantly lower than those of coliforms (F=38, *df*=1, p<0.001). The WWTP released enterococci into the river but it was not the only source.

Per volume, the bacteria abundance in sponges was generally higher than in water. For coliforms, the magnification factor was 160 – 1500, for enterococci 5 – 200. This showed the strong ability of sponges to retain bacteria.

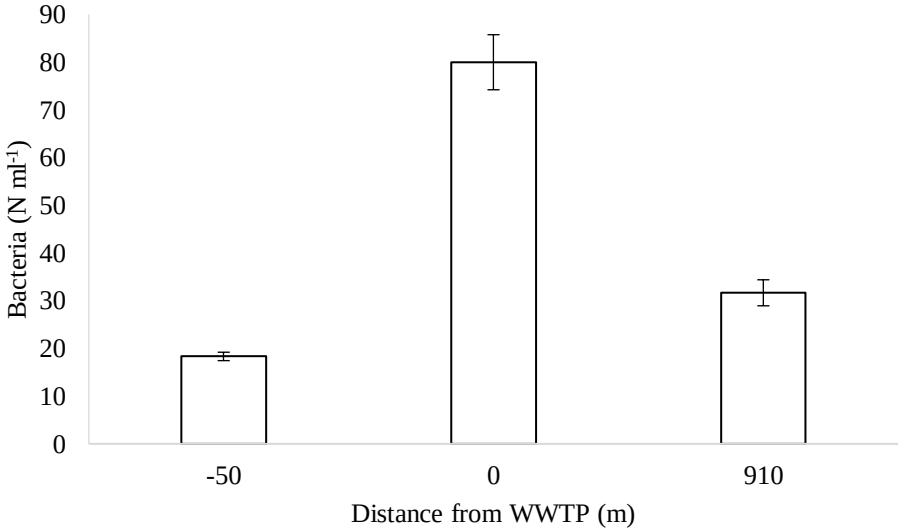



Figure 4.8b. Arithmetic mean and standard error values of enterococci in water samples with different proximity to the WWTP effluent discharge point. Site –50 m was upstream of discharge point. No significant differences in bacteria counts.

These results demonstrated that enterococci and coliforms were retained in all sponge samples showing the potential of sponges to be used as biomonitors for microbial water quality. The main findings of this research were:

1. Sponges retained coliforms in higher numbers than *Enterococcus* spp.
2. Sponges retained less *E. coli* and *E. faecalis* when they were exposed to high abundances of these bacteria than when they had exposure to lower abundances
3. The bacteria in sponges reflected exposure beyond the last 24 h.

For the field investigations, the main findings were:

4. The bacterial abundance in sponges were similar across a river transect within the individual rivers.
5. Abundance of bacteria in sponges varied between rivers.
6. The bacteria in sponges did not reflect the distance from a point pollution source, but the WWTP was not the only source of faecal indicator bacteria.
7. The abundance of coliforms and *Enterococcus* spp. in sponges did not reflect bacterial presence in the water at the time of collection.

## 4.4 Discussion

Sponges demonstrated that they can retain bacteria from the water thus reflecting the microbial water quality over a longer time-period. There is potential for the sponges to replace spot sampling methods used to estimate coliforms (in particular *E. coli*) and *Enterococcus* spp. abundance in water. Conventional spot sampling methods which can

be applied to microbiology, only detect the bacteria in the water passing at the specific moment in time that the sample was taken (Kirchner *et al.* 2004; Briciu-Burghina *et al.* 2014). However, the sponges can detect episodic microbial pollution. Current techniques to monitor water pollution were based on water chemistry sampling methods (Kirchner *et al.* 2004; Briciu-Burghina *et al.* 2014; Shore *et al.* 2017), but as sponges interact with the bacteria, their use as biomonitors would be based on natural biological interactions. If sponges were used to monitor pollution for faecal indicator bacteria, as in this study, enterococci could be monitored. However, specific *E. coli* monitoring should replace that of coliforms, as coliforms form a broad group of bacteria which contains both environmental and faecal bacteria (Noble *et al.* 2003).

The application of sponges to monitor microbial water pollution is discussed below, along with an evaluation of the experiments conducted. Bacterial content in the water and the potential for bacteria to infect sponges are discussed. The biomonitoring potential of the sponge varies with feeding, retention and the lifecycle of the sponge. The final part addresses the future developments needed before application of sponges for biomonitoring.

*Bacteria in sponges related to abundance and exposure*

The interactions of these filterfeeding organisms with bacteria, a food source, are well established (Reiswig 1975; Willenz *et al.* 1986). Sponges were widely known to concentrate bacteria from the water into higher abundance within their bodies (De Goeij *et al.* 2008; Stabili *et al.* 2008; Topçu *et al.* 2010). Stabili *et al.* (2008), for example, recorded faecal coliforms at the abundance of 1.20 MPN $g^{-1}$ from the sponge *Hymeniacidon perelevis* while these were 0.1 MPN $ml^{-1}$ from the water. This filtration ability resulted in Pile *et al.* (1997) finding a zone depleted in bacteria immediately above sponges, thus demonstrating the ability of sponges to alter the bacteria abundance in the water. Studies have shown no selectivity in the feeding ability of sponges (Pile *et al.* 1996; Wehrl *et al.* 2007). Wehrl *et al.* (2007) found that the marine sponge *Aplysina aerophoba* did not vary its filtering capabilities for six bacteria of different shapes and sizes including *Vibrio* and *Pseudoalteromonas*. However, in the current study, sponges always retained more coliforms than enterococci even when laboratory trials had higher relative abundances of *E. faecalis*. The difference in selectivity could relate to the bacterial species as Wehrl *et al.* (2007) did not use enterococci and they also carried out experiments in a 2 l volume compared to the 20 ml in this study which may have reduced the

sponge/bacteria contact diluting the effect of selective feeding. The reasons for different retention rates of bacteria were unclear, but it is unlikely to be due to higher feeding rates on *Enterococcus* spp. as sponges do not show selectivity in feeding (Wehrl *et al.* 2007). However, the work of Gardères *et al.* (2015) found that the marine sponge *S. domuncula* detected specific LPS from *Endozoicomonas, Pseudoalteromonas* and *E. coli* which were used for an immune response whereby sponges expressed macrophage genes. These sponges appear to have a specific recognition system for Gram-negative bacteria. This may explain the different sponge behaviour with *E. faecalis* and will limit the use of sponges for quantitative microbial sampling as they retain some bacteria better than others.

The lower retention of *E. faecalis* throughout and *E. coli* at 90% bacterial ratio were possibly a result of sponge infection. Sponges can succumb to bacterial infection where their cells become overrun with bacteria causing death to the organism with initial infection indicated by the suppression of 14-3-3 genetic markers (Böhm *et al.* 2001; Fu *et al.* 2013). Fu *et al.* (2013) demonstrated the infection of the marine sponge *H. perleve* with *Vibrio* spp. through genetic markers for cell death and its prevention including *Caspase* and 2 *Bcl-2* homology proteins. However, they found that *E. coli* did not infect sponges. Many studies have monitored the filtering ability of sponges with *E. coli* (Reiswig 1975; Willenz *et al.* 1986; Milanese *et al.* 2003) which would not have been possible if this bacterial group negatively affected sponges. Therefore, sponge infection by *E. coli* was also unlikely in this present study. No information on sponge infection from *E. faecalis* could be found to determine whether these bacteria were pathogenic to sponges. A sick sponge would also have limited potential to indicate microbial water quality as filtration ceases with poor health (Milanese *et al.* 2003). The immune system of sponges can be weakened by stress factors including rising temperature and eutrophication which allows for infection (Webster 2007; Kaluzhnaya & Itskovich 2015). Kaluzhnaya & Itskovich (2015) stated that the freshwater sponge *Lubomirshkia baicalensis* can become bleached whereby it loses symbiotic algae, a pandemonium which can also be associated with disease.

*E. coli* and *E. faecalis* both have virulence mechanisms (Jett *et al.* 1994; Sussman 1997) which would allow for infection of sponges. Enterococci for example attach to epithelial cells and release pheromones including *asa1* on successful attachment causing further aggregation (Zheng *et al.* 2017). If numbers were sufficient as indicated by quorum

sensing regulators, the bacteria could have entered a virulence mode through the release of pheromones triggering formation of biofilm and thus causing infection (Arias & Murray 2012). Successful attachment of bacteria would depend on the surface properties of both the bacteria and an attachment surface (Zhang *et al.* 2015); this would also apply to bacteria adhesion to sponges. However, even if *E. faecalis* does not infect sponges, using the aforementioned attachment, it could reduce sponge filtering capabilities by binding to cDNA (complementary DNA) immune markers for cell death and its prevention including *Caspase* and 2 *Bcl-2* homology proteins within the sponge cells thus reducing filtration (Wiens *et al.* 2004). *E. faecalis* virulence relies on surface adhesion before they can infect the host (Jett *et al.* 1994). If infection by enterococci was an actual threat, there could be an evolutionary advantage in the ability to reduce the content of these bacteria which may explain their low retention by sponges in this study.

It is possible to use sponges to extend the time-period for microbial water quality sampling. The bacteria in sponges were not replaced by more recent exposure, but instead, sponges represented the bacteria present in water from both exposure periods. However, they did not indicate which pollution event was most recent, only that a pollution event had occurred. No other studies investigating the timescale that sponges retain bacteria were found. However, seasonal changes of bacteria in sponges have been observed to reflect the microbial content of the water (Perea-Blázquez *et al.* 2013). *Haliclona venustina* showed higher feeding rates on bacteria in summer and winter, but fed mainly on the cyanobacteria *Prochlorococcus* in autumn as identified by flow cytometry (Perea-Blázquez *et al.* 2013). However, the timescale over which the microbial community inside the sponge changed was not investigated. A bacterial clearance rate study found filtering sponges released unwanted bacteria into the water within 3 h (Reiswig 1975), which may offer insight into the rate at which the sponge microbial community can undergo changes.

*Bacteria in sponges related to river and proximity to pollution source*

The work of Stabili *et al.* (2008) also focused on the ability of sponges to detect microbial pollution using *Vibrio,* faecal coliforms and faecal streptococci abundance in sponges to compare polluted and less polluted areas. *Spongilla officinalis* specimens living in proximity to a fish farm were compared to such samples from a site within a marine protected area. These sites were sampled at two different time periods and showed differences in the abundance of *Vibrio,* faecal coliforms and faecal streptococci over time

and between sampling sites. The viable counts of *Vibrio*, for example, varied in the marine protected area with $1.6 \times 10^2 – 1.7 \times 10^3$ cfu $g^{-1}$ in sponges, but remained stable at the fish farm between sampling periods with $1.2 \times 10^4$ cfu $g^{-1}$ in sponges 25 m from the fish farm and $1.2 \times 10^5$ cfu $g^{-1}$ in sponges directly below the fish farm (Stabili *et al.* 2008). This indicates the potential of sponges to act as biomonitors for microbial pollution as demonstrated in this study.

The bacteria in the sponges showed uniformity across a single profile in a river. The small rivers used for this study had continual mixing across the profile and so sponges could be sampled from anywhere across a transect to detect the microbial water quality. However, this uniformity may not apply to larger rivers. Quilliam *et al.* (2011) investigated the *E. coli* concentration across a Welsh river (River Conwy) finding significantly different bacterial abundances across transects. This river was larger, up to 1000 m wide while Cavan River was 4-6.5 m wide which is likely to affect water mixing. The *E. coli* abundance in the Welsh study varied from around 0.5–5.5 cfu $ml^{-1}$ (Quilliam *et al.* 2011). This cannot be compared to the current study which recorded total coliforms, but downstream of the last sponges, there were 696 cfu $ml^{-1}$. Part of this could be due to bacteria settling from the surface water to the sediments (discussed below) while Quilliam *et al.* (2011) took their sample from 1 m below the surface. Nevertheless, the River Conwy showed less water mixing, possibly related to river width. It is also possible that sponges enhanced the mixing of the water in the current study. Water mixing after all is important for providing sponges with a continual food supply. Without water mixing, Pile *et al.* (1996) found a 1 m zone with depleted bacteria and cyanobacteria immediately above sponges, but this will only affect sponges in lakes and not those in the flowing waters found in rivers. Therefore, water mixing is important for sponges, but it also has implications on water sampling as poorly mixed waterbodies may provide under or over estimates of aquatic bacteria.

This study found differences in bacterial abundance between rivers, probably due to differences in bacterial loading within a specific river. Catchment size and the human population density were two of the main factors affecting the abundance of faecal indicator bacteria in rivers (Crowther *et al.* 2010), and thus can result in bacteria loads specific to each river. Different bacteria sources and hydrological pathways could also result in different microbial assemblages in aquatic environments, however, globally, similar bacterial taxa including *Klebsiella* and *E. coli* were found in rivers and lakes

(Zwart *et al.* 2002) and thus the presence of bacterial groups may be similar, while their abundance will vary. Some bacterial groups appear to be more common than others with *E. coli* being more abundant than, for example, *Enterococcus* spp., *Klebsiella* spp. and *Citrobacter* spp. (Cabral 2010). This will also affect the quantity of different bacterial groups in sponges but their ability to concentrate bacteria from the water will probably mean that even rarer bacteria could be sampled with sponges.

The bacteria loading does not only vary between rivers, but can also do so within one river due to the impact of specific pollution sources (Ibekwe *et al.* 2011). It was hypothesised that the WWTP in Cavan would act as a bacterial pollution source because of previous evidence of the site failing water quality post treatment, thus providing the opportunity to investigate if the sponges' bacteria content was correlated with a pollution source. However, the bacteria in the main river channel was already high possibly due to inputs from the town centre, so this was not possible. A field containing around 20 cattle was also present alongside the river from 300–500 m downstream of the WWTP. Although the cattle did not have direct access to the river, depending on the field drainage, they could have supplied the river with faecal indicator bacteria and thus formed part of the unquantified diffuse source of coliforms and enterococci (Stumpf *et al.* 2010). This was not tested, but the bacteria in sponges collected from the site adjacent to the field were similar across the transect, suggesting inputs were from upstream sources and not from the field. This means that it was difficult to detect the impact of sponge retention in this study when the bacteria input at base level could not be fully quantified.

In addition to the effects of microbiological inputs, the water conditions also affect the survival of bacteria. The survival of *E. coli* in rivers has been demonstrated to be affected by water chemistry (McFeters & Stuart 1972). As water chemistry is site specific, this may have affected bacterial survival in the different rivers and so altered the bacteria abundance and composition to which sponges were exposed. Although there were many different inputs of bacteria, generally faecal indicator bacteria have a short lifespan in river water with *E. coli* inputs reducing to 0.2% of the original abundance in 5 d (McFeters & Stuart 1972). This survival also reduced with increasing river temperature where less than 50% of the original bacteria count were quantified after 24 h in water at 20 ℃ (McFeters & Stuart 1972). This was a surprising result as these bacteria originate in the gastrointestinal tracts of warm blooded animals, and their ideal growth temperature is around 35 ℃ and reaches a minimum at 8 ℃ (Ratkowsky *et al.* 1982; Scott *et al.* 2002).

As the water sampled in this study was at 18 ℃, the temperature may have reduced numbers of *E. coli,* or at least hindered their growth. This could help to explain the small number of coliforms, particularly in the WWTP effluent after effective treatment. This indicates that only more recent bacterial inputs will be available for sampling in sponges.

The enterococci abundance was higher in the WWTP effluent than in the river channel. Although WWTPs with tertiary processing can be efficient at removing nutrients and particulate matter to which bacteria can bind (Baudart *et al.* 2000; Hübner & Jekel 2013; Rajasulochana & Preethy 2016), most do not have specific treatment e.g. UV sterilization to remove bacteria. This means that some bacteria including those with antibiotic resistance are typically released from WWTPs into rivers (Sidrach-Cardona *et al.* 2014). However, the coliform numbers released from the WWTP on the Cavan River were lower than in the channel. Ibekwe *et al.* (2011) found *E.* coli, coliforms, enterococci and total bacteria to be higher in an urban river than in effluent from two WWTPs. Their study took place over two years indicating that diffuse sources of faecal indicator bacteria from agricultural and urban sources had a higher contribution to riverine bacteria than WWTP effluent discharge or point sources. Location, flow conditions and time of sample collection can have implications on the abundance of faecal indicator bacteria in water, but Ibekwe *et al.* (2011) found location had the highest contributing effect to bacterial abundance. This will have a direct impact on microbial water monitoring, but the use of regular monitoring sites may help to reduce this variability.

Bacteria abundance shows temporal and spatial variation in water, but sampling over 24 h found different patterns between water and sponges. Decreasing microbial abundance of coliforms or enterococci in the water was found with increasing abundance in sponges. This could indicate that the sponges were purifying the water by removing increasing levels of bacteria i.e. higher filtration activity means more bacteria in sponges, but fewer bacteria in the water. Therefore, allowing for the detection of the sponge filtering effect whereby they remove bacteria from the water. There is also the possibility for bacteria to be affected by other organisms in the river. Plant detritus was abundant on the river beds, some bacteria could have settled onto this substrate. Between the river sites at 100 and 400 m downstream of the WWTP there was also a large strand of an unidentified aquatic plant which grew to around 1 m in length. Rimes & Goulder (1985) found that aquatic bacteria could attach to submerged plants at a rate of $1.7 \times 10^4$ cfu $cm^{-2}$ $h^{-1}$ which could have removed aquatic bacteria in this study.

Sponges were not the only cause of reductions in the abundance of bacteria, as there are several other factors that can reduce the quantity of bacteria in water. These include the settling of bacteria on sediments, bacteria being bound to small suspended particles, and the damage or death of bacteria by UV radiation in areas of open, shallow water (Baudart *et al.* 2000; Stumpf *et al.* 2010; Briciu-Burghina *et al.* 2014). Rivers even at low temperatures have a rapid drop in bacterial number over time as McFeters & Stuart (1972) recorded a reduction in viable *E. coli* from $10^8$ to a minimum of $10^4$ cfu $ml^{-1}$ in 5 d with 4-6 ℃ river water. The bacteria can also be reduced by other organisms as bacteria are consumed by many organisms including protozoa, blackfly larvae and caddis larvae (Bick 1973; Baker & Bradnam 1976; Trimmer *et al.* 2009). All these processes would affect the bacteria abundance in the water, but their contribution to removal would be site specific.

*Further developments needed before sponges are used as a biomonitor of microbial water quality*

This study demonstrated the use of sponges to monitor water quality for two bacterial groups. It has generally been found that the microbes retained in sponges used in these types of studies were representative of those in the surrounding water including the *Prochlorococcus* sp. picoplankton and heterotrophic bacteria (Pile *et al.* 1997; Ribes *et al.* 1999; Wehrl *et al.* 2007). Therefore, it is likely that sponges can be used to detect several different bacteria, cyanobacteria and diatoms, as long as they occur in the water (Ribes *et al.* 1999). However, there are many issues which need to be addressed before sponge sampling can be widely applied to bacteria monitoring. For a quantitative biomonitoring, laboratory studies need to establish the sponges' reaction to known levels of bacteria over specific time-periods. By understanding the natural variation in the uptake of bacteria in relation to aquatic abundance and the time of exposure, it may become possible to estimate bacterial loading from the microbial analysis of sponge tissue samples from monitoring sites.

Sponges contain symbiotic bacteria (and algae) which need to be better quantified, as they could hinder the biomonitoring potential of sponges especially if the specific bacterial group form part of the natural sponge symbiont community in river conditions. Other studies have examined the symbiotic bacteria communities in *E. fluviatilis* and *S. lacustris* which contained symbiotic phyla including Proteobacteria, the phylum to which *E. coli* belongs (Gernert *et al.* 2005; Costa *et al.* 2013). However, no Firmicutes, the phylum

which includes *Enterococcus* spp. were found in their freshwater sponges. This means that sponges could contain symbiotic coliforms, but they do not appear to contain symbiotic *Enterococcus* spp., so any enterococci in the sponges had been filtered from the water. The presence of symbiotic coliforms in sponges needs to be further investigated, before they can be used for monitoring waterborne coliforms.

Other factors which would affect the use of sponges as biomonitors include the response of sponges to silt and storm flows and the seasonality of sponges. Both factors result in the ceasing of the filtration activity and the loss of the function as a biomonitor. This can be a response to touching (by e.g. suspended particles) where the sponges contract as a protection mechanism, which is likely to prevent them from filtering during storm events (Elliott & Leys 2007). In flood conditions scouring of the river bed can also lead to sponge fatalities as flood removed all sponges from sites sampled by Pronzato & Manconi (1995), but they recolonised within seven months. Sponges are also sensitive to silt (particles <63 µm) which can kill sponges within 70 days as silt blocks their canal system (Maldonado *et al.* 2008; Bell *et al.* 2015). This would prevent them from being used as biomonitors in river systems prone to extensive inputs of fine sediment or storm events.

Freshwater sponges are seasonal and die back into gemmules during unfavourable conditions (Paduano & Fell 1997; Hill & Hill 2002). This means that in the UK and Ireland they cannot be used to sample bacteria during winter. However, this would not affect their use in bathing water microbial sampling as this is usually carried out over the spring and summer months (Environment Agency 2016). Beneficially, gemmules allow sponges to be hatched under laboratory conditions; these specimens would then be placed in a river specifically to sample the bacteria. Therefore, they can be moved to specific locations and exposed to the water for specific time-periods, thus providing time-integrated information on bacteria presence in the water. However, the sponges must be kept in such a way as to prevent accidental introduction to the river being sampled, if they are not already present within the catchment. Growth of sponges from gemmules with reduced microbial content offers a potential avenue to further explore the use of sponges as biomonitors for microbial water quality. These sponges could be placed into a river for a specific time-period, so the internal bacteria would better indicate the microbial water content. As sponges represent only one group of filterfeeders, it is possible that similar monitoring schemes could be developed with other organisms, e.g. freshwater mussels.

## 4.5 Summary

This study demonstrated the potential of sponges to be used for biomonitoring of coliforms and enterococci. The bacterial numbers in the sponges were always higher than in the water and demonstrated a higher time-period for bacterial detection. *E. faecalis* retention was lower than that of *E. coli*. Bacteria in river sponges varied more between rivers than across one river, reflecting different bacterial regimes. The abundance of bacteria in either the water or the sponges was not related to the distance from the WWTP effluent discharge point as this was only a minor bacterial pollution source. Sponges can be used as a biomonitor in rivers for the presence of coliforms and enterococci, but additional research is required if they are to provide quantitative results or to be used for monitoring other bacterial groups.

# 5. Do freshwater sponges facilitate the transfer of antibiotic resistance in waterborne *Enterococcus faecalis*?

This chapter assesses the experimental evidence for the facilitation of antibiotic resistance transfer between Gram-positive bacteria. Laboratory experiments exposed sponges in microcosms to strains of *E. faecalis* with a resistance to either vancomycin or rifampicin. Live and dead sponges were used to determine if successful gene transfer resulted from active filtration by sponges or surface attachment of bacteria. The novelty of this section was testing the facilitation of conjugal transfer between bacteria by sponges.

## 5.1 Introduction

Antibiotic resistance of nosocomial bacteria is a problem within a clinical setting and perceived threat to the environment (Berendonk *et al.* 2015). The transfer of ARG can potentially impact on both human and animal health (Aminov & Mackie 2007; Flores Ribeiro *et al.* 2014). Widespread use of antimicrobial and antibiotic compounds has contributed to a rise in resistance to these chemicals, making treatment with antibiotics less effective (Davies & Davies 2010; Marti *et al.* 2014). Bacteria can develop antibiotic resistance through selective pressure, and several mechanisms of acquiring resistance genes from other microbial organisms have been identified (Dzidic & Bedekovic 2003; Wilson & Salyers 2011). The most significant of these mechanisms in terms of the amount of mobilised DNA is conjugal transfer which requires direct contact between donor and recipient bacteria (Massoudieh *et al.* 2007; Wilson *et al.* 2010).

*E. faecalis* can acquire ARG through pheromone-induced conjugal transfer (Clewell & Weaver 1989). Increasingly, isolates from this species show high levels of antibiotic resistance (Arias & Murray 2012; Conwell *et al.* 2017). Antibiotics against which resistance has been recorded include ciprofloxacin, erythromycin, rifampicin, and vancomycin (Arvantidou *et al.* 2001; Arias & Murray 2012). Enterococci resistant to vancomycin are of greater clinical concern due to high patient mortality and also due to the epidemiology whereby these isolates are spreading to other countries (Emaneini *et al.* 2016). VRE account for 8.5-10.8% of the enterococci infections in the UK from general medical and intensive care respectively (Brown *et al.* 2008). Resistance to vancomycin can be plasmid bound and therefore transfer to other bacteria can occur through conjugal transfer (Conwell *et al.* 2017).

Aquatic ecosystems are one of the main reservoirs for ARB along with soil (Wright 2010; Marti *et al.* 2014). Potential hotspots for the occurrence of ARB within aquatic ecosystems include discharge points for effluent from WWTP plants and farm runoff

(Michael *et al.* 2013; Sidrach-Cardona *et al.* 2014; Berendonk *et al.* 2015). Both are likely ARB hotspots, because they contain bacteria and sublethal concentrations of antibiotics. WWTPs expose environmental bacteria and those discharged from the intestines of warm blooded organisms to a dilution gradient of at least partially unmetabolized antibiotics from excretions of animals and humans that have received these chemotherapeutic agents in medical treatment (Michael *et al.* 2013). The antibiotics in wastewater included ofloxacin, sulphamethoxazole and trimethoprim, and ARG included *sul1, bla*$_{TEM}$, and *ermB* (Subirats *et al.* 2017). In a study focusing on just one group of ARB, VRE and their *vanA* genes have been found in 32 of the 37 wastewater samples collected by Oravcova *et al.* (2017). This prevalence of VRE further indicates these organisms' potential as shown in conjugal transfer of this resistance to other bacteria.

There is some evidence for the occurrence of conjugal transfer in natural environments, e.g. in aquatic biofilm and the intestines of flies that contained high numbers of ARB (Massoudieh *et al.* 2007; Akhtar *et al.* 2009; Doud *et al.* 2014). However, it remains poorly understood which environmental factors and processes may contribute to increased antimicrobial resistance transfer (Berendonk *et al.*, 2015). In a laboratory setting, filter mating is used to facilitate conjugal transfer between bacteria (Ghosh *et al.* 2011; Haug *et al.* 2011; Doud *et al.* 2014). The filtration process aggregates aquatic bacteria on a solid surface to increase the probability of successful gene transfer. For natural environments Lupo *et al.* (2012) have suggested a similar facilitating role by aquatic filterfeeders, which would concentrate waterborne bacteria, thereby enabling conjugal antibiotic resistance transfer. However, there still does not appear to be any experimental evidence for this stipulation. As sponges are filterfeeders, they have the potential to facilitate conjugal transfer by bringing bacteria into direct contact within their confined filter chambers. Facilitation of conjugal transfer between bacteria by sponges can be tested with single species assays or genomic approaches.

This chapter investigated the potential of the freshwater sponge species *E. fluviatilis*, as a model filterfeeding organism, to facilitate the conjugative transfer of vancomycin resistance between waterborne bacteria of two different *E. faecalis* strains. No other experimental studies addressing the facilitation of antibiotic resistance transfer by filterfeeders have been found.

*Aim and objectives*

The aim of this section was to investigate the potential of freshwater sponges to facilitate the transfer of ARG between two *E. faecalis* strains.

The objectives were:

1. Assessment of effects of sponges on transconjugant numbers in their ambient environment.
2. Comparison of transconjugant numbers in live and dead sponges to assess the impact of active filtration.

## 5.2 Methods

Sponges and controls were exposed to two different strains of *E. faecalis* which were compatible for pheromone-induced conjugal transfer (Conwell *et al.* 2017). Experiments were conducted with live and dead sponges, with controls for each (Figure 5.1). Conjugation on an agar plate was also conducted with each experiment set to provide a control of transfer efficiency with a standard method.

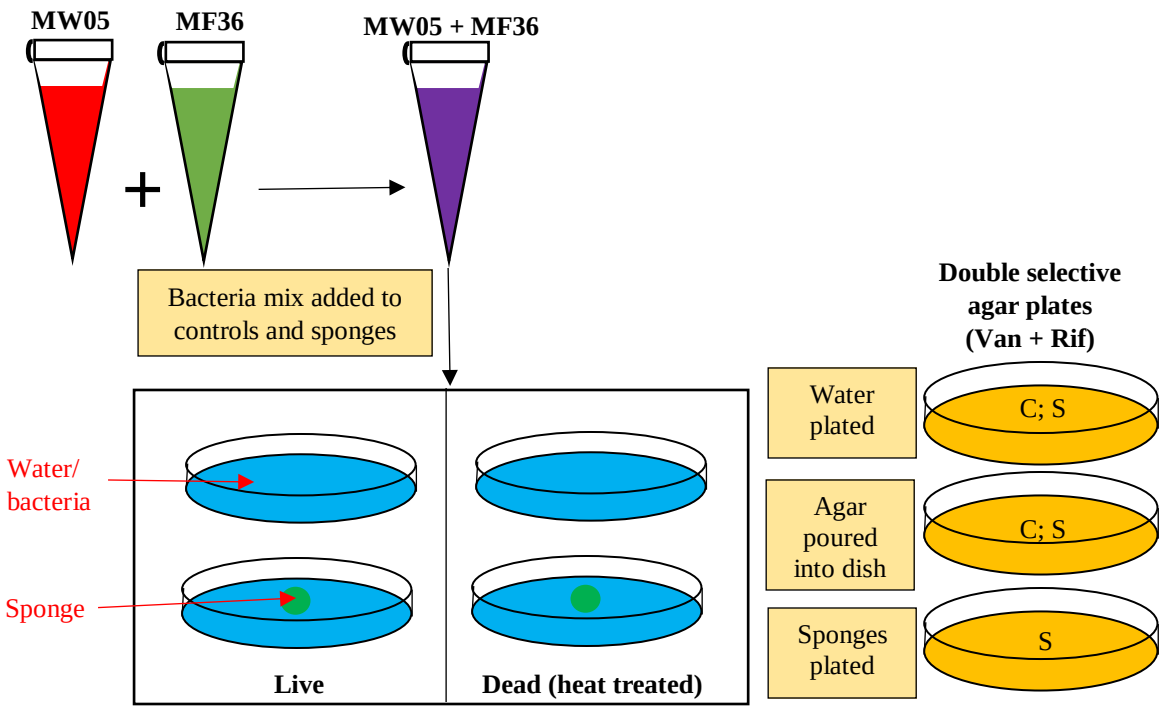



Figure 5.1. The experimental protocol to test if sponges facilitate conjugal transfer of antibiotic resistance between *E. faecalis* where MW05 = *E. faecalis* (rifampicin resistance), MF36 = *E. faecalis* (vancomycin resistance), van = vancomycin, rif = rifampicin, C = control, S = sponge.

Gemmules from Cavan River were treated with 1% $H_2O_2$ for 10 min and stored in sterile water at 4 ºC until needed. For sponge microcosms, gemmules were hatched onto the base of a sterile glass petri dish with 10 ml of UV treated (254 nm, 10 min) mineral water at

20 ºC, while control dishes contained only water. Half of the sponges had been hatched two days early so that these sponges could be killed before the trial by a 48 h exposure to a temperature of 35 ºC. Half of the control dishes had also been subjected to this temperature before the trial. Before their use in the trial horizontal dimensions of 7-day-old sponges on individual dishes were measured to determine their surface area.

*Experimental Protocol for conjugal transfer*

Overnight bacterial cultures of *E. faecalis* of donor strain MF06036$^{Van}$ and recipient strain MW01105$^{Rif}$ (Conwell *et al.* 2017) were grown in TSB (Oxoid). Both strains were grown separately for a further 90 min in fresh TSB at a tenfold dilution. After mixing at a 1:14 donor to recipient ratio, the mixture of bacterial strains was tenfold diluted in UV treated mineral water. Water from petri dishes of all microcosms and controls was replaced by 20 ml of the diluted bacteria mixture before a 24 h incubation at 20 ºC.

Water from these petri dishes was transferred to universal tubes and vortexed. 1 ml of liquid from each replicate was spread on a double selection plate of Tryptone Soya Agar (TSA; Oxoid) with 100 µg ml$^{-1}$ rifampicin and 10 µg ml$^{-1}$ vancomycin (both from Sigma). After 48 h incubation at 37 °C colonies were counted as presumed transconjugants.

Sponges were lifted with sterile pipettes or tweezers and placed into Eppendorf tubes with 200 µl of autoclaved water. Eppendorf tubes were vortexed for two minutes and 100 µl of the undiluted sponge cell suspension was plated onto a double selection plate for incubation and counted as above. Tenfold serial dilutions were carried out with the remaining 100 µl of sponge cells for plating on single antibiotic plates to allow the quantification of each parent in the sponge. The petri dishes from the experiment were washed three times before the addition of double selection agar. Plates were incubated as above. Experiments were completed on 30 dishes of each type (control, heated control, live *E. fluviatilis*, or dead *E. fluviatilis*).

*Parent numbers for conjugation reaction and control*

Serial dilutions were carried out with the 90 min cultures of recipient and donor strains. These were placed onto TSA and incubated for 48 h at 37 °C to calculate the parent numbers which were available for conjugation. Both parent strains used for the 90 min culture were also streaked on a double selection agar to ensure that no contamination had occurred before the trial.

*Conjugation control on agar plates*

This followed the method from Conwell *et al.* (2017). After the 90 min culture and mixing of the parents at a 1:14 ratio ($MF06036^{Van}$: $MW01105^{Rif}$), 800 µl aliquots were plated onto two TSA plates. Both were incubated for 24 h, one at 20 °C and the other at 37 °C. The bacteria were scraped off the agar and lifted into an Eppendorf tube, where they were resuspended in 1 ml phosphate buffered saline (PBS). Contents of each tube were diluted and homogenised. 100 µl aliquots were plated onto a double selection plate, incubated and counted as before.

*Calculations for transconjugants and transfer efficiency*

From plate counts, the number of transconjugants on the dishes and number of transconjugants in 1 ml water were obtained. The number of transconjugants per dish was counted if below 500 and estimated if they exceeded this. These values provided transconjugant numbers in dishes and in water. To obtain the number of transconjugants in the sponge, the following calculation was used:

$$T_S = \frac{(PC \times W)}{(DF \times SHD)} \qquad \textbf{Equation 5.1}$$

where $T_S$= transconjugants per sponge, PC= plate count on agar, W=portion of water added to sponge, DF=dilution factor i.e. 100-fold, SHD=sponge size as $mm^2$ horizontal measurement.

The number of donor bacteria retained in the sponges at the end of the experiment was used to calculate the transfer efficiency as follows:

$$D_S = \frac{Dml}{(DR \times DW)} \qquad \textbf{Equation 5.2}$$

where $D_S$=number of donor bacteria per sponge, Dml=donor per 1 ml, DR=dilution in recipient bacteria, DW=dilution in water

To calculate the efficiency of the conjugal transfer in sponges, equation 5.1 was divided by equation 5.2, i.e. $T_S$ divided by $D_S$.

The agar controls were not diluted in water, so the transconjugants and donors added were calculated using a modified version of equations 5.1 and 5.2:

$$T_C = \frac{Tml}{DF} \qquad \textbf{Equation 5.3}$$

Where $T_C$=transconjugants per agar plate, Tml=transconjugants per ml, DF=dilution factor

$$D_C = \frac{Dml}{DR} \qquad \textbf{Equation 5.4}$$

where $D_C$=donor per agar plate, Dml=donor per 1 ml, DR dilution in recipient bacteria

These values were used to calculate the transfer efficiency on the agar plates by dividing equation 5.3 by equation 5.4, i.e. $T_C$ divided by $D_C$.

*Data visualisation and statistical analysis*

Data for the transconjugants on dishes, in the water and in sponges were visualised individually as Tukey style box-whisker plots for each treatment showing the median, lower quartile, upper quartile, range, and outliers. All statistical analysis was completed in SPSS (IBM v22). The data for the transconjugants on the dishes were not processed by statistics as they contained estimate values. The data for each treatment (control, live sponge, heated control, dead sponge) and measurement (water or sponge) were tested for normality using the Kolmogorov–Smirnov test. The data was not normally distributed and so non-parametric tests were applied to all data. The Kruskal-Wallis test was used with a significance level of 0.05. As post-hoc tests pairwise comparisons between groups were carried out with Mann-Whitney U tests and Bonferroni correction.

## 5.3 Method development

*Establishing temperature range for conjugation*

Before conducting the trial, it was necessary to establish the viable temperature range for gene transfer because the reported temperature optimum for the bacteria of 37 ºC exceeded the active temperature range of sponges (Jackson *et al.* 2005). This temperature pilot test involved the method described under *conjugation control* (5.2). Plates were incubated at 4, 10, 20, 37 ℃ with five replicates for each temperature treatment. Conjugation occurred from 4–37 ℃ (Table 5.1), and the transfer efficiency increased with temperature. For the sponge trials 20 ℃ was chosen, as sponges survived at this

temperature for more than a week (see appendix 2), and conjugation efficiency appeared sufficiently high for the detection of transconjugants.

Table 5.1. Arithmetic means of transconjugant numbers and transfer efficiency on agar after a 24 h incubation at a range of temperatures where T/D = Transconjugant to Donor ratio

| Temperature (°C) | Transconjugants | T/D |
|---|---|---|
| 4 | 1 | $2.1 \times 10^{-10}$ |
| 10 | 28 | $1.5 \times 10^{-8}$ |
| 20 | 5,040 | $2.7 \times 10^{-6}$ |
| 37 | 43,928 | $2.3 \times 10^{-5}$ |

*Trials in sponges - Universal tubes, live and dead sponges*

Prior to the use of universal tubes, other preliminary trials were conducts. The results are summarised in appendix 4. Live and dead sponges were used for these trials. Live sponges could be detected by monitoring daily growth. To determine if heat treatment killed sponges, water filtration was used. Filtration was tested by adding a drop of ink or *E. coli* GFP to the water. Live sponges turned black with ink or fluoresced under UV light with the *E. coli* GFP. Neither ink nor fluorescent bacteria were observed with the heat-treated sponges, which was taken as evidence for this method's efficacy in killing sponges.

Preliminary trials were carried out in universal tubes with live sponges (*S. lacustris* and *E. fluviatilis*). Their results showed that both species of live sponges had significantly higher numbers of transconjugants than the controls, and that there were no significant differences between the two species of sponges. It was assumed that the conjugal transfer was occurring due to the sponges concentrating bacteria as they filtered the water column. Therefore, the trial was repeated with dead sponges (killed by temperature). Similarly, there was not a significant difference between either species of dead sponges, or between the controls and sponge. Comparison of the controls between live and dead sponge trials were significantly different, but the same method was used ($U=563$, $p<0.001$). This led to the conclusion that the observed pattern may not reflect the impact of sponges, but relate to the bacteria themselves.

Further tests with controls showed that bacteria were adhering to the plastic tubes causing unpredictable background conjugation to occur. Attempts to remove the attached bacteria by washing did not remove adhering bacteria from the plastic tubes. Further investigation of controls identified that bacteria could be washed from glass petri dishes allowing for

quantification, as only a few transconjugants were found on the pertri dishes at the end of the experiment. As there had been no significant differences between the sponge species, all further trials were conducted on *E. fluviatilis* only.

5.4 Results

*E. faecalis* with either vancomycin or rifampicin resistance were mixed in water and placed into control petri dishes, and sponge microcosms with live or dead sponges. 24 h later the transconjugant bacteria with resistance to both antibiotics were quantified from the water, petri dish surface and the sponges. Sponge controls without exposure to *E. faecalis* strains did not show any growth on double selection plates. The parent isolates also did not show any growth on double selection plates and so growth on these plates with the treatments were from gene transfer. Contradictory to the method development studies, the number of transconjugants on the glass surfaces was high and so most counts were estimates. The number of transconjugants on the dishes were lower in the heated control dishes than on the dishes for the other treatments where most plates had over 2000 transconjugants (Figure 5.2).

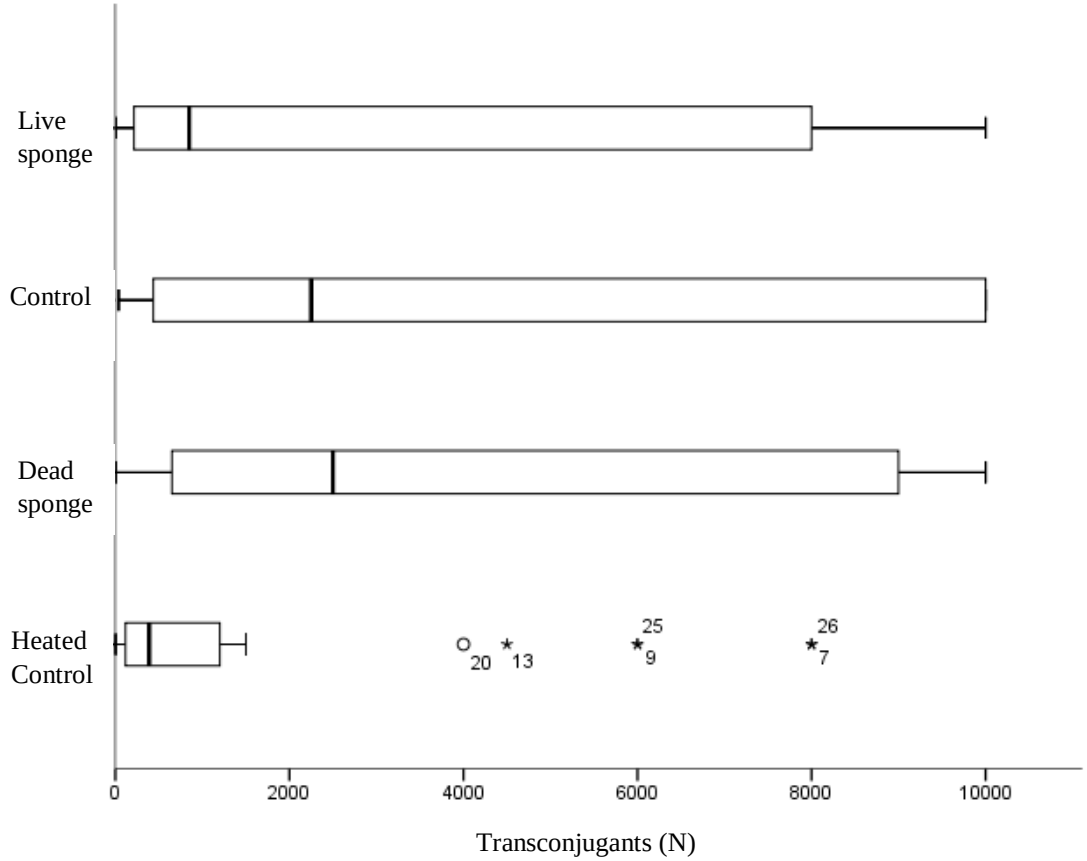



Figure 5.2. Transconjugants on the glass surface from controls and sponge microcosms.

In water the number of transconjugants was generally higher when a sponge was present and slightly higher with live sponges (Figure 5.3). There were generally fewer than 25 transconjugants per 1 ml of water in all treatments. No transconjugants were recorded in the water in some replicates from all treatments, but this occurred more frequently in

control water than from sponge microcosm water. There was a significant difference in the number of transconjugants in the water (H=14, p=0.005). Pairwise comparison showed that the live sponges were significantly higher than both controls (controls- U=13, p=0.005; heated controls U=661, p=0.001) and that the dead sponges were significantly higher than the heated controls (U=588, p=0.035).

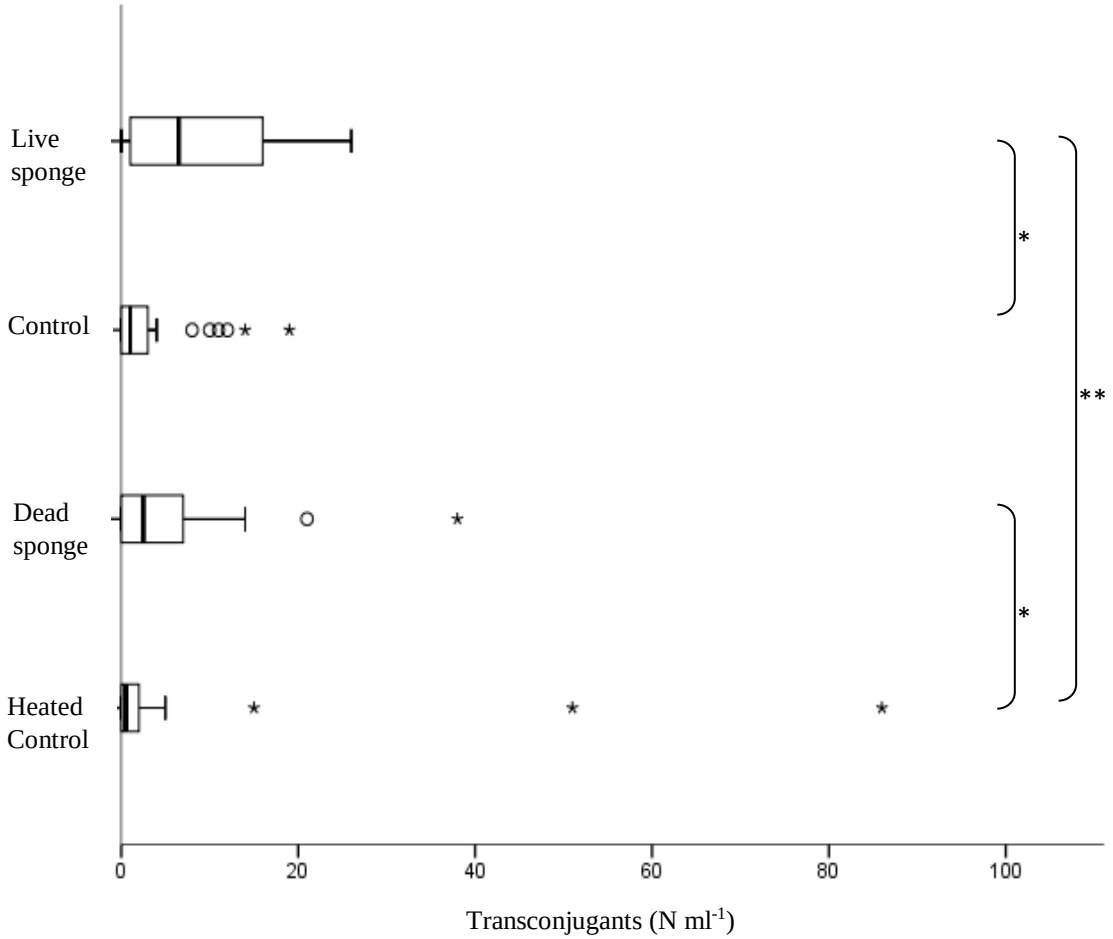



Figure 5.3. Transconjugants in the water from the different treatments. * = $p < 0.05$, ** = $p < 0.01$, all other results not significant.

The number of transconjugants in or on the sponges was similar for both live and dead sponges generally with fewer than 10 transconjugants per $mm^2$ (Figure 5.4). This difference between sponges was not significant. There was also no significant difference in the number of recipient or donor bacteria isolated from the live to the dead sponges and live and dead sponges retained similar concentrations of *E. faecalis* (U=21, p=1.000).

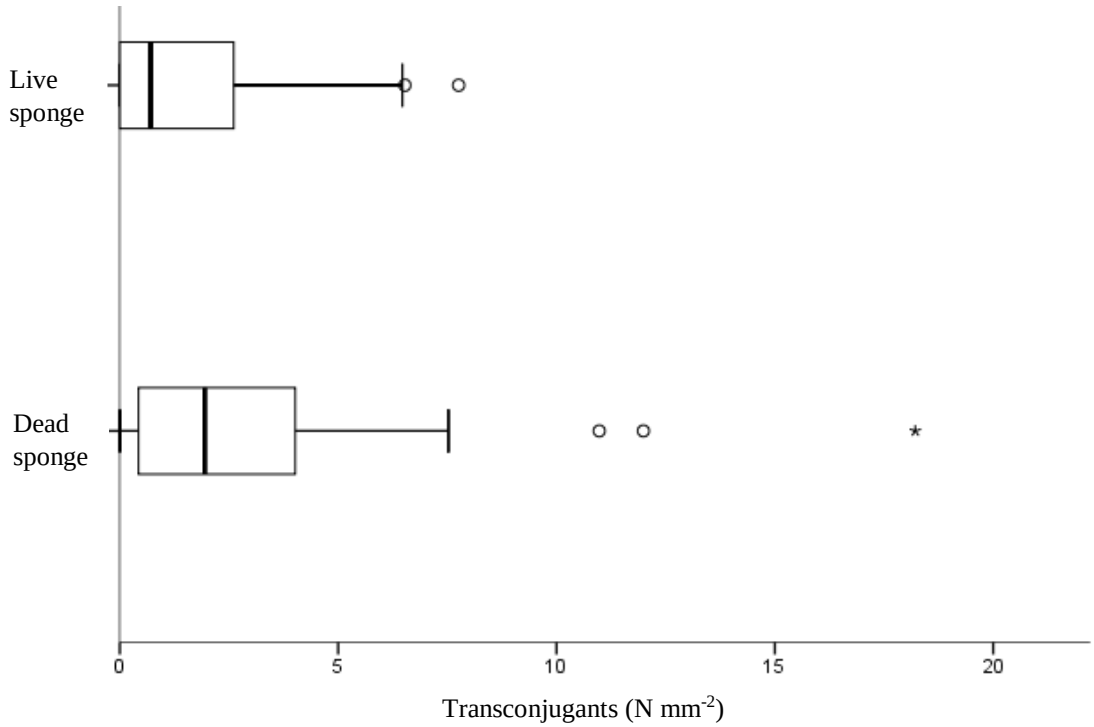



Figure 5.4. Transconjugants in the live and dead sponges, the difference was not significant.

The transfer efficiency in the sponges was lower than on agar (Table 5.2). In sponges, there was a maximal transfer efficiency of $10^{-6}$ but this was ten times lower than on the agar plates at this temperature. Both the mean and maximal transfer efficiency was similar with the live and dead sponges, similar to the results shown in Figure 5.4. The transfer efficiency on the agar plates also increased with temperature and was tenfold higher when the temperature was increased to 37 ℃.

Table 5.2. The arithmetic means and standard error value (SE) and the maximum transfer efficiency of the transconjugant: donor ratios for the sponges and agar controls.

| | | **Transconjugant: Donor** | |
|---|---|---|---|
| **Sample** | **Treatment** | **Mean ± SE** | **Maximum** |
| Sponge | Live sponge (20 ℃) | $3.3 \times 10^{-7} \pm 7.4 \times 10^{-8}$ | $1.4 \times 10^{-6}$ |
| | Dead sponge (20 ℃) | $7.3 \times 10^{-7} \pm 1.9 \times 10^{-7}$ | $4.1 \times 10^{-6}$ |
| Agar control | 20 ℃ | $2.8 \times 10^{-5} \pm 1.5 \times 10^{-5}$ | $7.7 \times 10^{-5}$ |
| | 37 ℃ | $3.7 \times 10^{-4} \pm 8.2\ 10^{-4}$ | $8.2 \times 10^{-4}$ |

This study demonstrated that sponges allowed for conjugal transfer of antibiotic resistance between waterborne *E. faecalis*. The main findings were:

1. The presence of sponges increased the number of transconjugants in the water.
2. The number of transconjugants in and on sponges was similar for live and dead sponges, indicating that the conjugal transfer was not due to filtration.

## 5.5 Discussion

Molecular biological characterisation by Conwell *et al.* (2017) of the *E. faecalis* strains used in this study has provided convincing evidence for conjugative transfer of vancomycin resistance between donor and recipient bacteria. Therefore, in this study, bacterial colonies with phenotypic resistance against both antibiotics on double selection plates were presumed to be transconjugants. Elevated numbers of such bacteria in water from sponge microcosms indicated that the presence of freshwater sponges was associated with conjugal gene transfer of antibiotic resistance. However, the lack of significant differences between microcosms of live and dead sponges in microbial analysis of water and sponge cell suspensions suggested, that active filtration might not a be significant contributing factor.

Bacteria conjugal transfer associated with sponges was thought to be linked to sponge filtration, as filter mating is known to enhance transfer efficiency within a laboratory setting (Ghosh *et al.* 2011; Haug *et al.* 2011; Doud *et al.* 2014). Active filtration by sponges were thought to concentrate bacteria necessary for the transfer (Clewell & Weaver 1989) into the confined canals of the sponges. However, the results of this study indicated that conjugal transfer was more likely to be related to the sponge surface than active filtration. Collagen-binding protein has previously been identified by Daniels (2011) on cells of both *E. faecalis* strain used in this study, which enhanced its virulence potential. The collagen-binding protein, *ace* was found by Daniels (2011). This protein allowed for the attachment of enterococci to collagenous material. As sponges are made up of spongin, a variation of collagen, this may enhance bacterial adhesion to the sponge surface. In the marine sponge *Rhopaloeides adorabile* the α – Proteobacterium NW4327 kills sponges using an enzyme which digests collagen (Mukherjee *et al.* 2009). If the spongin structure of the sponge can be attached by a collagen specific enzyme, the *ace* protein in the *E. faecalis* are also likely to be attracted to the sponge surface. This may offer explanation for the sponges retaining similar abundances of *E. faecalis*, regardless of whether they were alive or dead. Once *E. faecalis* can adhere to a surface, they can release aggregation substances that cause conjugal transfer (Jett *et al.* 1994). Alternatively, to direct individual cellular attachment, the sponge surface may have allowed for a biofilm to develop where conjugal transfer occurred. Biofilms have previously been shown to facilitate conjugation between bacteria (Massoudieh *et al.* 2007; Cook *et al.* 2011). Their formation can be rapid with *E. faecalis* biofilm forming

overnight on microtiter plates (Zheng *et al.* 2017), and so its formation on sponges over the 24 h experiment is plausible.

In addition to conjugal transfer in biofilm, it can also occur between planktonic cells in the water but at a much lower efficiency (Sengeløv & Sørensen 1998; Shu *et al.* 2013). In stream water, maximal transfer efficiency of $2.5 \times 10^{-4}$ were recorded between *E. coli* compared to $2.1 \times 10^{-1}$ with filter mating (Sengeløv & Sørensen 1998). In the current study, conjugal transfer also occurred at a low rate in the sponge-free controls but aquatic transconjugants in the presence of sponges were higher. This indicated that transconjugants can detach from the sponge surface, or that sponges caused aggregation of bacteria in the water. With evidence that sponges contain antimicrobial molecules (Marinho *et al.* 2012), it is possible that their body (live or dead) released bioactive compounds into the water thus repelling bacteria from the sponge and causing aquatic aggregates where conjugal transfer took place. However, this remains to be tested. This could have implications for occurrence of ARB in freshwater ecosystems especially as sponges have a wide biogeographic range throughout much of the northern hemisphere (Økland & Økland, 1996).

This study demonstrated the association of sponges with bacterial conjugation in a laboratory setting; similar processes could occur in the field. The *E. fluviatilis* gemmules were collected downstream of a WWTP where ARB are known to occur in high numbers (Doud *et al.* 2014). At the locations mentioned above, sponges can therefore be exposed to ARB, so the sponge associated conjugal transfer observed in this trial could also occur in their natural environments. This could provide one explanation for sources of newly emerging ARB in aquatic ecosystems (Berendonk *et al.* 2015). As river water is used for drinking water and crop irrigation, these bacteria could potentially enter the food web as observed with *E. coli* O157: H7 (Solomon *et al.* 2002). This would provide a link to the clinic where ARB from the water move into sponges for subsequent gene transfer and their release results in human infection.

The results of this study indicated that conjugal transfer rates were similar in live and dead sponges. This also meant that the sponges were not feeding on the bacteria, or that feeding had no net effect on transfer efficiency. As the conjugal transfer was not facilitated by filtration, it was possible the transfer efficiency would reduce with sponge feeding, however this was not observed. Filterfeeding sponges have been observed to

remove up to 74% of the waterborne bacteria passing through their canals for consumption or retention within the sponge (Pile *et al.* 1996) but this did not occur with *E. faecalis*. However, sponges contain a wide range of other bacteria which could potentially exchange genetic material. Thomas *et al.* (2016) recorded a high diversity of bacterial phyla from sponges with individual specimens containing 13-41 phyla. *E. coli*, for example, are retained in a higher abundance than *E. faecalis* (Chapter 4) and might serve as another test case for conjugal transfer facilitation, especially because some *E. coli* exhibit multiple antibiotic resistance and conjugal transfer is possible between *E. coli* (Altherr & Kasweck 1982; Son *et al.* 1997; Heijnen & Medema 2006). This could also provide sources for new pathogens of clinical importance and so the above experiment should be repeated with conjugal compatible *E. coli* strains to investigate if conjugal transfer is associated or facilitated by sponges.

Although this study demonstrated conjugal transfer, transduction is also important for the transfer of antibiotic resistance in freshwater (Lupo *et al.* 2012). There is also evidence that transduction can occur with marine sponges. Webster & Thomas (2016) studied the microbiome in sponges and found evidence of transduction in some of the isolated bacteria from the marine sponge *Amphimedon queenslandica*. Therefore, the acquisition of antibiotic resistance in bacteria associated with sponges could occur with different transfer mechanisms.

*Comparison of transfer efficiency to other studies*

The transfer efficiency is affected by properties of individual bacterial strains and the environment where conjugation takes place. In the sponge environment, transfer efficiency was lower than observed in other organisms and with different bacteria. Bacterial conjugal transfer has been recorded in the gastrointestinal tract for a number of terrestrial organisms including cockroaches and house flies occurring with *E. coli, E. faecalis* and *Salmonella enterica* (Akhtar *et al.* 2009; Anacarso *et al.* 2016). Transfer efficiencies within such host organisms varied from $10^{-3}$ and $10^{-4}$ with house flies and cockroaches respectively (Akhtar *et al.* 2009; Anacarso *et al.* 2016). The transfer efficiency was lower in sponges which could be due to the dilution of the bacteria in water that did not affect the other studies. The efficiencies of transfer with sponges were also lower than recorded with filter or agar mating. Doud *et al.* (2014) and Conwell *et al.* (2017) demonstrated transfer efficiencies of $10^{-3}$ through agar mating; these were substantially higher than the $10^{-7}$ calculated for sponges in this chapter's experiments.

There are two potential causes; the bacteria were more concentrated on the agar plates, as they were not diluted in water, and agar provides a nutrient-enhanced medium designed to facilitate bacterial growth.

Temperature and the type of transfer plasmid also affect conjugation by changing the activity of bacteria. Published conjugation experiments took place at higher temperatures, usually around 37 ºC which were optimal for the selected bacteria groups (Ghosh *et al.* 2011; Conwell *et al.* 2017). In the present study, an agar conjugation control at 37 ºC achieved a maximum transfer efficiency 10 times higher than at 20 ºC which demonstrated the effect of temperature on transfer. Transfer efficiency can also vary depending on the type of plasmid being transferred even where the same bacteria strains and methods were applied. Ghosh *et al.* (2011) found transfer efficiencies between *E. faecium* varied with plasmid type and were higher for gentamicin resistance than streptomycin with an average transfer of $10^{-3}$ and $10^{-7}$ respectively. However, they did not identify the plasmid responsible for the transfer of each antibiotic resistance. Daniels (2011) and Conwell *et al.* (2017) have suggested that the plasmid responsible for the vancomycin resistance transfer observed with the bacteria used in the current study was the 70 kb plasmid pCF10. This plasmid contains the *agg* gene that causes aggregation of the bacteria as a response to pheromones and can also carry resistance to tetracycline and vancomycin.

*Limitations and future work for conjugal transfer*

The main limitations in this study were related to the age and size of the sponges. Gemmule-grown sponges covered a surface area of a few $mm^2$ and hence filtered less than adult sponges. Adult sponges were not used for these experiments as tested samples of these contained bacteria which grew on the double selection agar. Another limitation was the ability of the bacteria to adhere to the petri dish surface, which resulted in background conjugation that was not observed in the method development. These transconjugants could not be removed by washing.

Although the sponges did not show a relationship between filterfeeding and conjugal transfer in bacteria, the suggested facilitation of HGT by filterfeeders (Lupo *et al.* 2012) would need to be more extensively tested. It should be tested in other aquatic filterfeeders, with different bacteria and in adult sponges with a fully developed bacterial community. The trials in this study could be extended further by identifying the precise location where

conjugation occurs on the sponge, if sponges release compounds that enhance aquatic bacteria aggregation, and by investigating if transconjugants can detach from the sponge surface. If the occurrence of such facilitating processes in filterfeeders was verified, the next step in research would be to monitor the fate of multidrug resistant bacteria from these sources, because it is unknown whether filterfeeding organisms release such bacteria into ambient water or aquatic sediments.

## 5.6 Summary

This study appears to present the first experimental evidence that the presence of freshwater sponges was associated with bacterial conjugal transfer of antibiotic resistance. The transfer occurred with both live and dead sponges and so was likely to be the result of surface attachment of bacteria to sponges. The presence of sponges also increased the number of transconjugants in the water. These transconjugants could be from the sponge which are released or detach from the sponge surface. Alternatively, the conjugal transfer could occur in the water with sponge antimicrobial properties repelling bacteria to form aquatic aggregates. Sponges could therefore be a source of bacteria with multiple antibiotic resistance in aquatic environments. More research is needed to examine if other aquatic filterfeeders facilitate gene transfer in bacteria and if sponge filtration would facilitate the transfer of antibiotic resistance with other species of bacteria.

# 6. Antibiotic resistant bacteria from gemmules of the freshwater sponges *Ephydatia fluviatilis* and *Spongilla lacustris*

This section contains information on bacteria within or on the surface of gemmules from freshwater sponges *E. fluviatilis* and *S. lacustris*. The bacteria abundance in sponge microcosms was monitored for hatching and unhatched gemmules. Selected isolates were tested for antibiotic resistance to six antibiotics. The recovery of ARB from the gemmule surface has not been reported elsewhere.

## 6.1 Introduction

Bacteria are vital to sponges throughout their lifecycle providing them with nutrition and increased growth rates (Willenz *et al.* 1986). Bacteria also form symbiotic relationships with a wide variety of bacteria groups (Wehrl *et al.* 2007; Thomas *et al.* 2016). Similar to marine sponges, freshwater sponges appear to contain a wide array of symbiotic bacteria (Gernert *et al.* 2005; Keller-Costa *et al.* 2014). Included in the bacteria obtained from sponges are strains which exhibit antibiotic resistance (Selvin *et al.* 2009). However, the seasonal nature of freshwater sponges means that they cannot harbour bacteria throughout the entire year, unless bacteria are incorporated into their gemmules or those retained on gemmule surface are of sponge origin. Daphnia enter a similar dormancy and Mushegian *et al.* (2017) found that a symbiotic community was required for their survival upon hatching. It is unknown if bacteria are needed for the long-term survival of sponges, but there is evidence that freshwater sponges rely on bacteria for antimicrobial properties (Keller-Costa *et al.* 2014).

Gemmules are currently considered to be internally void of bacteria, despite evidence that algal cells can be incorporated into these structures (Simpson & Fell 1974; Willianson & Williamson 1979). This means that upon hatching, sponges must derive their symbiotic community from the water or gemmule shell. However, the theoretical possibility of a bacterial presence inside gemmules has been noted (Rozenfeld & Curtis 1980). If sponges do not incorporate bacteria into the gemmules, the fate of these symbiotic bacteria after the death of their host is unknown. The demise of the host is likely to affect the symbiotic bacteria which could be released into the water or form a biofilm on the gemmule surface. The release of ARB from dying sponges could be of clinical concern as potential feedback of bacteria from the environment to the clinic has been suggested (Berendonk *et al.* 2015). To identify the presence of bacteria inside the gemmules, disinfection of the surface was carried out before experiments to reduce bacterial contamination. The release of bacteria by hatching sponges was then monitored. Further tests sought to establish if the bacteria from gemmules exhibited antibiotic resistance.

*Aim and objectives*

The aim was to investigate if sponge gemmules were associated with bacteria and if these exhibited antibiotic resistance. The following two objectives were addressed:

1. Investigation of bacteria release from gemmules upon hatching.
2. Assessment of resistance to antibiotics of isolates from newly hatched sponges.

## 6.2 Methods

Bacteria were isolated from the controls and sponge microcosms and grown on agar (Figure 6.1). Randomly selected colonies were tested for antibiotic resistance to six different antibiotics.

*Experimental set up and bacteria testing*

Mineral water was UV treated (254 nm, 150 min) and filtered with a 0.22 µm syringe filter (Millex-GS). *E. fluviatilis* gemmules were collected from Cavan River and *S. lacustris* gemmules from Downhill River. In an adaptation of a surface-disinfection method from Rasmont (1970) gemmule surfaces were exposed to a solution with 1% $H_2O_2$ for 10 min and then transferred into Eppendorf tubes with 1 ml of water and kept at 20 ℃. On days 0, 1, 2, 4, 6, 8, and 14 (Figure 6.1), 50 µl water sample was removed and plated onto TSA with tenfold serial dilutions as needed for counting. Plates were incubated at 37 ℃ and counted after 24 and 48 h. This allowed for bacteria released upon hatching, or from the gemmules surface to be monitored. The water from control tubes containing no gemmules was also plated as described for the sponges. In total there were 15 *E. fluviatilis* (8 hatched), 30 *S. lacustris* (11 hatched) and 10 controls. A higher number of replicates of *S. lacustris* were used to ensure adequate hatching as these gemmules had shown lower hatching efficiency. The horizontal area covered by the hatched sponges and the size of the gemmule were measured at day 7 and 14 of the trial.

*Antibiotic resistance testing of isolated bacteria*

All plates with bacteria colonies from day 14 and randomly selected plates from day 1 and 6 were investigated for antibiotic resistance. Plates were examined from the start middle and end of the trials to establish if ARB were found throughout the experiment. Bacterial cells were sampled from multiple colonies per plate using a sterile loop (Figure 6.1). Cells were resuspended in 500 µl of PBS and vortexed. The suspension was streaked onto TSA agar containing one of the six antibiotics at concentrations from Table 6.1

which reflect the antibiotic susceptibility levels established by Daniels (2011) for aquatic enterococci. Enterococci were used for positive and negative controls for each antibiotic. These plates were incubated at 37 ℃ and examined for growth after 24 and 48 h. This was completed for a total of 75 plates. The bacterial suspension in PBS was also streaked onto MacConkey No. 3 and Slanetz & Bartley media to test for coliforms and enterococci respectively. These were incubated at 37 ℃ and examined for growth after 24 and 48 h. Any growth on a selective agar medium was also tested for antibiotic resistance to the same six antibiotics.

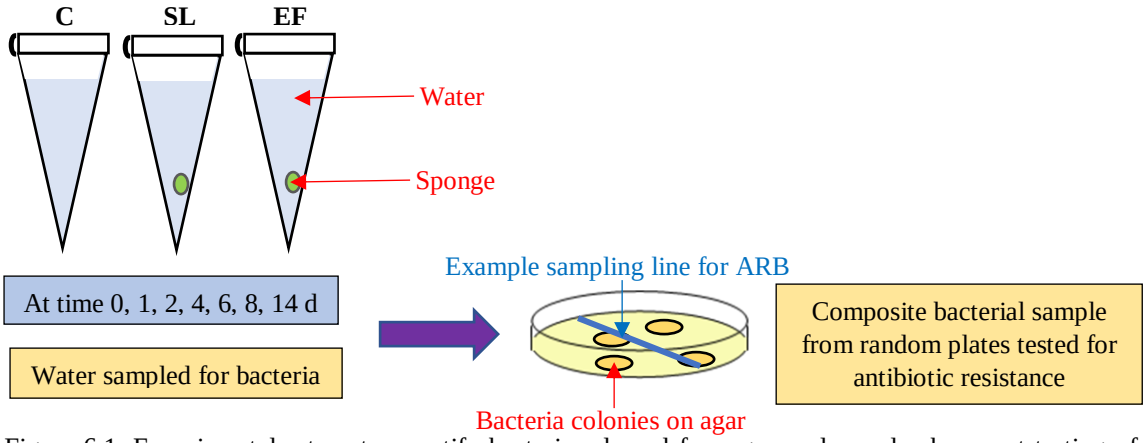



Figure 6.1. Experimental set up to quantify bacteria released from gemmules and subsequent testing of bacteria for antibiotic resistance where C = control, SL = *S. lacustris* and EF = *E. fluviatilis*, ARB = antibiotic resistant bacteria.

Table 6.1. Antibiotic concentration tested to establish if gemmules contained antibiotic resistant bacteria.

| **Antibiotic** | **Concentration (µg ml$^{-1}$)** |
|---|---|
| Ampicillin | 10 |
| Erythromycin | 32 |
| Rifampicin | 100 |
| Tetracycline | 16 |
| Trimethoprim | 10 |
| Vancomycin | 10 |

*Isolation and sequencing of fluorescent Pseudomonas*

One bacteria type isolated from the gemmules and sponges showed fluorescence under UV light. A pure culture was grown on King's B medium incubated at 20 ℃ for 48 h (Keller-Costa 2014). This culture successfully grew on Pseudomonas Isolation Agar (Sigma-Aldrich) but could not be identified beyond *Pseudomonas* sp. These bacteria were also tested for antibiotic resistance using the antibiotics in Table 6.1.

Characterisation with molecular biology methods was carried out by M. McCarron. The culture was extracted and amplified according to the protocol by Twigg (2016). The culture was boiled with 50 µl nuclease-free water for 2 min before centrifugation at 10,000 rpm. 1 µl was added to a mastermix containing 1 X buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 2.5 U *Taq* DNA polymerase, the required volume of water and 0.5 µM of each primer set. The primers used were 9bfm – GAGTTTGATYHTGGCTCAG and 1512Ur – ACGGHTACCTTACGACTT. This mix was used for PCR under the following conditions, initial denaturing – 94 ℃ for 3 min, 30 cycles with: denaturing – 94 ℃ for 45 s, annealing 52 ℃ for 30 s, extension - 72 ℃ for 135 s, and a final extension of 72 ℃ for 10 min. Gel electrophoresis was used to check for product, before the amplified sample DNA was cleaned in accordance to the Wizard SV Gel and PCR Clean-up System (Promega 2010). The sample was sequenced by GATC Biotech using the same primers as above and the additional primer 536f - GTGCCAGCMGCCGCGGTAATWC. Sequences from the three primer sets were stitched together and analysed to remove interference in DNA dragon (SequentiX 2010). The remaining sequence was entered into the NCBI nucleotide BLAST (NCBI 2017) to identify the species isolated.

*Data visualisation and analysis*

The arithmetic means and standard error values of bacteria abundance in the sponge/gemmule water from $mm^2$ surface were calculated for hatched and unhatched gemmules from each sponge species. These values were used to plot line graphs with a semi-logarithmic scale y-axis for each treatment to show the change in bacteria abundance over time.

The data for the bacteria counts in the gemmule/sponge were not normally distributed (Kolmogorov–Smirnov test) and so the Scheirer-Ray-Hare test was applied with a significance threshold of $p<0.05$. Replicates were compared between time and treatment (control, hatched *S. lacustris*, unhatched *S. lacustris*, hatched *E. fluviatilis* and unhatched *E. fluviatilis*). The Scheirer-Ray-Hare test was also used to compare the two sponge species regardless of hatching status. Another comparison involved hatched and unhatched sponges, regardless of species.

For the ARB, the percentage of total tested colonies from each sponge species exhibiting resistance to individual antibiotics was calculated for each analysis day of the trial. For each sponge species arithmetic means and standard error values of these percentages were

plotted in bar charts. The same also applied to the antibiotic resistant coliforms and enterococci, whose graphs were not included. The number of antibiotic resistance exhibited from each sample were also calculated and plotted as a bar chart.

## 6.3 Results

*Bacteria in gemmule/sponge microcosm with gemmule surface-disinfection*

The bacteria from surface-disinfected gemmules and hatching sponges were quantified, and selected isolates were tested for antibiotic resistance. Even after subjecting gemmules to the surface-disinfection treatment, a bacterial presence was detected on unhatched gemmules. Abundances were initially low but started to increase rapidly between day 2 and 5, after which a lag phase was reached (Figure 6.2). The highest values for arithmetic means of abundance were $2 \times 10^5$ cfu $ml^{-1}$ and $8 \times 10^6$ cfu $ml^{-1}$ for *S. lacustris* and *E. fluviatilis* respectively. The growth in bacteria was attributed to the hydrogen peroxide treatment releasing nutrients which facilitated bacteria growth. There was a significant difference between all treatments (control, hatched *S. lacustris*, unhatched *S. lacustris*, hatched *E. fluviatilis* and unhatched *E. fluviatilis*; SS/MS=159, *df*=4, $p<0.001$) and over time (SS/MS=40, *df*=5, $p<0.001$). However, the interaction between treatment and time was not significant (SS/MS=16, *df*=20, $p=0.691$) so all treatments responded similarly over time with increasing abundance of bacteria. Regardless of hatching status there was also a significant difference between the sponge species over time and the interaction between time and treatment (treatment- SS/MS=346, *df*=1, $p<0.001$; time- SS/MS=476, *df*=1, $p<0.001$; interaction- SS/MS=93, *df*=5, $p<0.001$) with higher bacteria numbers recorded for *E. fluviatilis*. Finally, there was a significant difference between hatched and unhatched sponges and over time (treatment- SS/MS=4.9, *df*=1, $p<0.027$; time- SS/MS=451, *df*=1, $p<0.001$) with higher bacteria numbers from unhatched gemmules, but the interaction was not significant (SS/MS= 10, *df*=5, $p=0.075$).

*Antibiotic resistance profiling of bacteria isolates from gemmules*

Pilot studies investigated the presence of antibiotic resistant enterococci in adult sponges using the same six antibiotics which were tested with gemmules. The adult sponges contained enterococci resistant to ampicillin, rifampicin, tetracycline, trimethoprim and vancomycin. This range of antibiotics was also tested on bacteria from gemmules, which had been collected from the same rivers as the adult sponges. All enterococci in positive controls grew on the antibiotic plates while all negative controls were inhibited. Tests for ARB involved 75 plates and in total, isolates exhibiting resistance against each of the six antibiotics were found (Figure 6.3). Only one of the isolated bacteria did not exhibit multi-antibiotic resistance with most isolates being resistant to three of four antibiotics (Figure 6.4). The most frequently identified traits were resistance to ampicillin, trimethoprim and vancomycin. Only a few plates contained bacteria resistant to tetracycline and only a single plate contained bacteria resistance to rifampicin. Antibiotic resistant had a higher

relative frequency among isolates from gemmules of *E. fluviatilis* than from those of *S. lacustris* with most exhibiting resistance to four and three antibiotics respectively. Isolates with resistance to all antibiotics was found at day 1, 6 and 14 of the experiment with similar resistance profiles seem on all days. The high standard error for *E. fluviatilis* isolates to erythromycin were attributed to only 28% of the colonies from day 1 showing resistance to this antibiotic which increased to more than 80% of the colonies at the other sampling periods.

Selective media found 5% and 2% of the tested plates contained coliforms and enterococci respectively. While coliforms were resistant to ampicillin and vancomycin, the single *Enterococcus* isolate from the gemmules showed resistance to all six antibiotics.

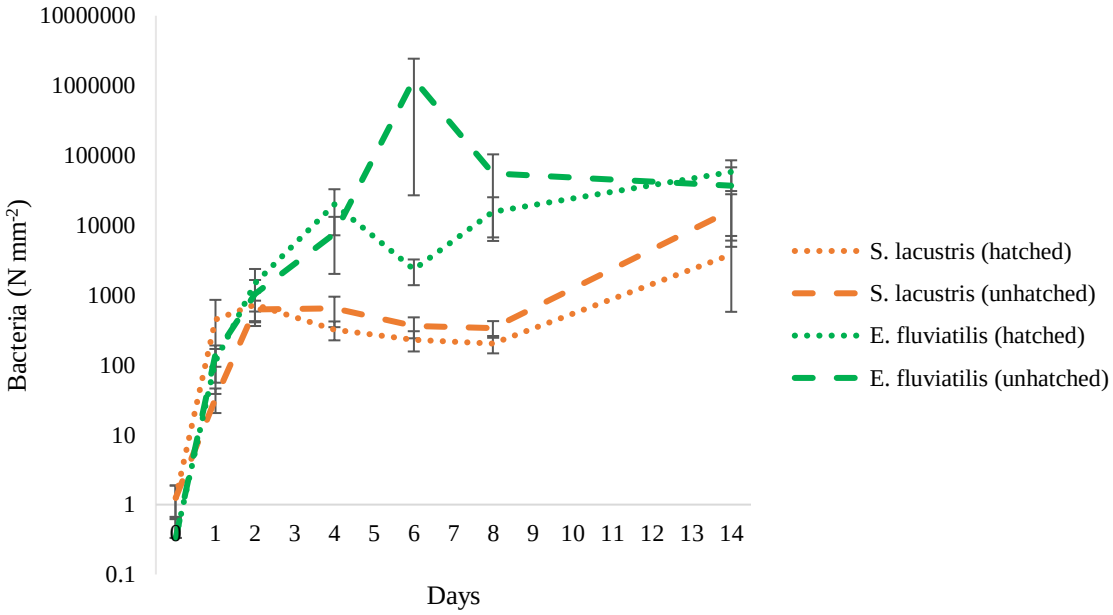



Figure 6.2. Semi-logarithmic plot of bacteria released into the water from gemmules and newly hatched sponges after surface-disinfection. Bacteria were quantified per $mm^2$ of gemmule/sponge horizontal dimension.

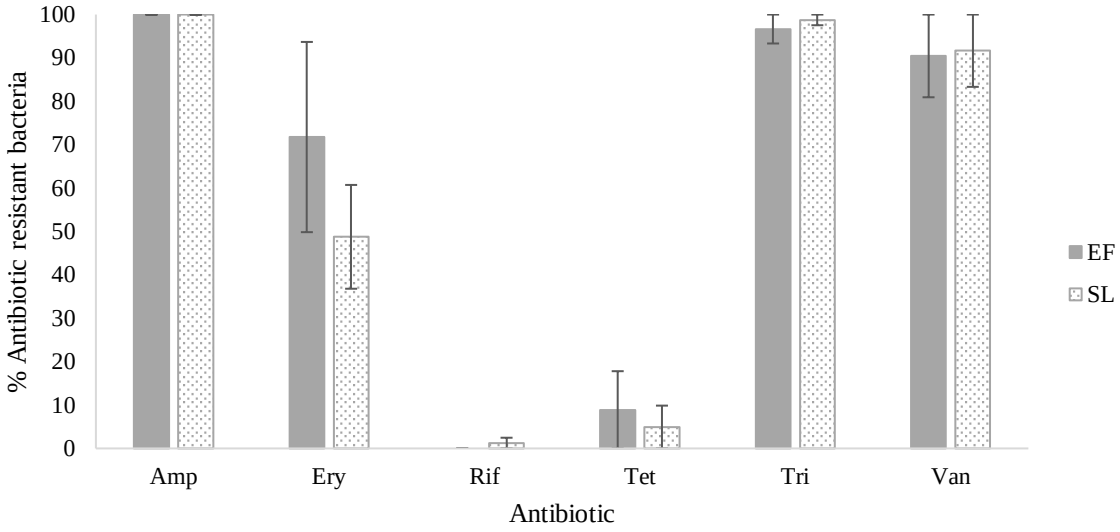



Figure 6.3. Arithmetic means and standard error values for relative frequency of antibiotic resistance among bacteria isolates from surface-disinfection gemmules of *E. fluviatilis* (EF) and *S. lacustris* (SL) where amp = ampicillin, ery = erythromycin, rif = rifampicin, tet = tetracycline, tri = trimethoprim, van = vancomycin.

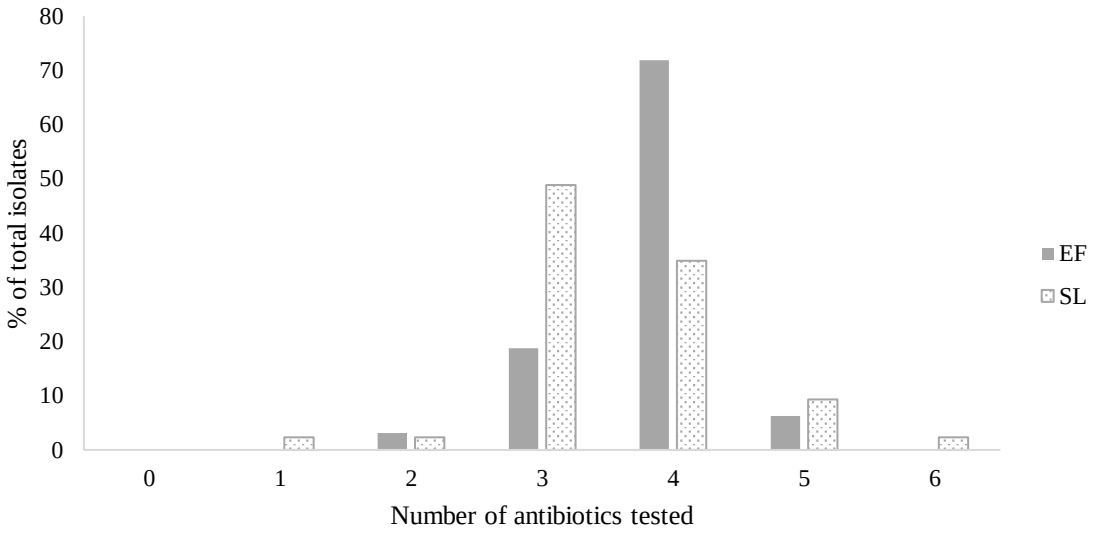



Figure 6.4. Frequency of antibiotic resistance among bacteria isolates from surface-disinfection gemmules *E. fluviatilis* (EF) and *S. lacustris* (SL).

*Fluorescent Pseudomonas from sponges*

The fluorescent isolate of *Pseudomonas* sp. could not be identified to species level, but the NBCI BLAST highlighted eight species with matching sequences (Table 6.2). This strain was resistant to ampicillin and trimethoprim. During the conjugation experiments (Chapter 5), bacteria of the same phenotype were found within all hatched sponges and water in their microcosms.

Table 6.2. Species of fluorescent *Pseudomonas* with sequences that yielded a 100% hit in a database search with NBCI BLAST in comparison to those isolated from sponges.

| **Species** | **Number of hits** |
|---|---|
| *P. vancouverensis* | 4 |
| *P. fluorescens* | 2 |
| *P. umsongensis* | 2 |
| *P. mohnii* | 5 |
| *P. putida* | 3 |
| *P. jessenii* | 3 |
| *P. corrugate* | 1 |
| *P. reinekei* | 1 |

The main findings from the above results were:

1. Hydrogen peroxide treatment did not remove all bacteria from the gemmules and so, it could not be identified whether the exterior gemmule surface or the inside of gemmules were the source of origin for the isolated bacteria.
2. Bacteria associated with the gemmules all showed multiple antibiotic resistance.

## 6.4 Discussion

The abundance of bacteria on the gemmule surface further indicates the strong association of sponges with their microbial community. This could be related to either the lifecycle of the sponge or the surface adhesion of bacteria. Bacteria may be retained on the surface of the gemmule for four main reasons: host protection, food supply, bacteria aggregation/ attachment or the natural settling of bacteria on a surface. These are discussed below. Among the bacteria retained on the gemmules were ARB which may have environmental impacts or simply reflect the bacteria present in the water. These topics are discussed below along with an evaluation of the efficiency of the surface-disinfection of the gemmules and the antibiotic resistance profile of the bacteria isolates.

Bacteria on the gemmule surface may be advantageous to newly hatched sponges for defence against infection, or as a potential food source. Mushegian *et al.* (2017) showed the importance of bacteria to daphnia after dormancy whereby the symbiotic community was vital for their existence and daphnia without bacteria on or in their ephippial case died within 7 d. This community came from the water, external surface of the ephippial case and inside the ephippium. The bacteria which showed a high association with the daphnia ephippial case included the phyla proteobacteria and actinobacteria which have also been associated with sponges (Costa *et al.* 2013; Mushegian *et al.* 2017). One

symbiont of vital importance to sponges could be *Pseudomonas*. It has a strong association with sponges and typically is the only bacterial group with permanent presence in sponges kept in laboratory conditions (Böhm *et al.* 2001). The *Pseudomonas* sp. appears to contribute to sponges' antimicrobial properties against bacteria and oomycetes (Keller-Costa *et al.* 2014). Keller-Costa *et al.* (2014) isolated 90 fluorescent *Pseudomonas* from *E. fluviatilis* and further tests showed that half of these inhibited bacterial growth, 35% inhibited protozoan and 32% inhibited oomycetes potentially from the synthesis of molecules including pyoluteorin or hydrogen cyanide. Therefore, the incorporation of these types of bacteria in sponges or gemmules may reduce the abundance of other microorganisms. Some environmentally isolated *Pseudomonas* sp. can produce phenazine which prevents fungal growth (Tupe *et al.* 2015), thus indicating the complexity of the symbiotic relationships found within sponges or their gemmules and their need for bacteria which inhibit other microbes. In the current study these bacteria provided insight into bacteria transfers between hatching sponges and ambient water. These bacteria were found both within the sponge and the water after hatching indicating that bacteria can be incorporated into the hatching sponge, or released from the gemmule surface to the surrounding water. This could indicate that sponges selectively retain bacteria on their gemmules which will prevent infection when they hatch. However, there is also potential for bacteria on the gemmule surface to cause infection in newly hatched sponges.

There was a higher bacterial abundance with the *E. fluviatilis* gemmules than the *S. lacustris.* This difference is unlikely to be species specific but instead it may relate to the site of collection as these sponges as adults contained similar bacteria, mainly from the phyla actinobacteria (Gernert *et al.* 2005; Costa *et al.* 2013). However, different bacterial abundances were found in adult sponges between rivers (Chapter 4). Even within one catchment, there can be a wide variation in bacterial loads, but they are similar at one site over time (Ibekwe *et al.* 2011). It may be possible to better quantify bacteria on the gemmule surface through the use of imaging techniques such as the scanning electron microscope. Imuta *et al.* (2008) used the scanning electron microscope to view biofilm production in *E. coli* on intestinal mucus and so this method could be adapted to allow better understanding of how the bacteria coat the gemmule surface.

The abundance of bacteria on the gemmule surface could also be explained by responses in bacteria. Bacteria carry out quorum-sensing to communicate with each other whereby

they can create biofilms or cause virulence (Zheng *et al.* 2017). It is possible that the bacteria create a biofilm on the gemmule surface as a response to the demise of their host by expressing biofilm proteins such as *asa1*, *cylA* and *agg* which has been found to enhance biofilm production in *E. faecalis* (Zheng *et al.* 2017). As an alternative to the surface bacteria originating from the sponge, it is also possible that the bacteria on the sponges came from the water. Particles including bacteria settle out of the water column, often onto sediment (Liao *et al.* 2014). This settlement is also likely to occur onto bottom-dwellers such as sponges. Although adult sponges have some ability to avoid being covered by settling particles, such mechanisms are not available to the inactive gemmules.

It is also possible that the bacteria form a biofilm on the gemmule surface which is disrupted through surface-disinfection. Santiago *et al.* (2016) found that hydrogen peroxide treatment at a concentration of 12 µM was able to prevent the formation and to disrupt pre-established biofilms of *P. aeruginosa*. This effect was observed with concentrations well below those used in this study which were 330, 000 µM and so considerable disruption to the biofilm on the gemmule could be expected. This could have resulted in bacteria being released into the surrounding water which were detected in this study. The surface-disinfection could also have resulted in the release of molecules into the water that could facilitate bacterial growth, but evidence for this process has not been obtained. This may also explain the stagnation in bacterial growth after 2-4 d whereby the source of nutrients for bacterial growth may have been fully utilised.

*Efficiency of surface-disinfection of gemmules*

As a resting stage for the diapause in the life cycle of freshwater sponges, gemmules theoretically offer an opportunity to remove exterior microbes from an organism through chemical disinfection. This would not be possible in experiments with marine sponges or other filterfeeders without such a robust resting stage. Chemical treatments can kill filterfeeders like some invasive mussel species and are therefore sometimes used to prevent their spread, although mussels are capable of avoiding temporary exposure through closing their shell (Aldridge *et al.* 2006). Contrary to tests by Rasmont (1970) who developed the method, in this study, the hydrogen peroxide treatment was not potent enough for a complete inactivation of surface bacteria. Rozenfeld & Curtis (1980) also found that bacteria persisted on the gemmule surface after a hydrogen peroxide treatment (7.5% for 5 min). Other studies have not noted sponge contamination with bacteria post gemmule treatment with hydrogen peroxide, but some referred to it as a method to reduce

bacteria and fungi (Funayama *et al.* 2005; Karlep *et al.* 2013) and did not state that bacteria were completely inactivated.

The bacteria numbers recovered from the treated gemmule surfaces in this study were greater than those determined from untreated gemmules by Rozenfeld & Curtis (1980) who found 100 cfu $ml^{-1}$ when 120 gemmules were hatched in 1 ml of water, while the current study would have found bacterial abundance in the region of 1.2 x $10^5$ and 1.2 x $10^6$ cfu $ml^{-1}$ from 120 gemmules. Rozenfeld & Curtis (1980) also found bacteria only appeared in the water 3 d after hatching. It is unclear why this study recorded a much higher bacterial abundance. The bacteria regime in the rivers where the gemmules were formed may provide a potential explanation, but this remains to be tested. To remove surface contamination, Rozenfeld & Curtis (1980) found that a combination of hydrogen peroxide treatment (7.5% for 5 min) with sodium hypochlorite (1% for 2 min) was effective in killing bacteria. Therefore, an application of this disinfectant combination would perhaps also have been effective in stopping bacterial growth from exterior gemmule surfaces in this study thus allowing for bacteria from inside the gemmule to be detected.

Contrary to expectations, the hatched sponges did not show higher bacterial abundance than unhatched gemmules. However, this does not mean that bacteria were not released from inside the gemmule. Rozenfeld & Curtis (1980) were also unable to determine if gemmules contained bacteria, but they did not rule out this possibility. It is also possible that symbiotic bacteria incorporation varies with individual specimens as Sheikh-Jabbari *et al.* (2014) did not find symbionts inside the daphnia ephippial cases but in their later study, these were found in some cases (Mushegian *et al.* 2017). To detect the bacteria, from inside the ephippial cases the external surface was treated with 5% hypochlorite before hatching and subsequent sequencing of the bacteria within. This method could also be applied to the gemmules and after successful surface-disinfection, PCR could amplify bacterial 16S DNA from the gemmules' interior, to detect and identify any bacteria that might indeed be incorporated into these structures.

*Evaluation of antibiotic resistance profile of bacteria from gemmules*

The antibiotic resistance of bacteria varies with individual strains and environmental exposure to antibiotics. Adult marine sponges contained ARB resistant to ampicillin, erythromycin, and tetracycline (Selvin *et al.* 2009; Hoppers *et al.* 2015). However, it

appears that previous studies have not tested for ARB on gemmule surfaces. While the symbiotic bacteria assemblage in marine sponges has been described as stable throughout the seasons (Hoppers *et al.* 2015; Pita *et al.* 2016), the seasonality of freshwater sponges was thought to limit these organisms' interactions with bacteria to the growing season. This study, however, showed that gemmules could harbour ARB on their surfaces over the winter. Therefore, the seasonal nature of freshwater sponges would not result in the removal of potential clinically relevant bacteria from the sponge system as they are likely to form part of the adult sponge symbionts upon hatching. They would also not be removed from the aquatic ecosystem and they could also be released into the water when the sponges hatch as demonstrated with Pseudomonas. This is likely to occur with other bacteria as well and provides evidence that sponges can release ARB into the environment.

The antibiotic concentrations used were based on the work of Daniels (2011) who determined the inhibitory concentrations for enterococci from rural Irish streams. These breakpoints may not apply to other bacteria. Breakpoints used for antibiotics can also vary between laboratories as demonstrated by Smith *et al.* (2009) for florfenicol, oxytetracycline and oxalinic acid. Their study demonstrated that sometimes antibiotic concentrations in these tests can be insufficient to detect resistant bacteria and that incorrect classifications of wild type strains with no acquired resistance as non-wild type exhibiting clinical resistance have occurred (Smith *et al.* 2009). The antibiotic breakpoints used in this study were unlikely to be affected by this source of error as bacteria did not grow in negative controls, but these were all enterococci and so may not apply to other bacteria groups from the sponges.

Most gemmules were associated with bacteria resistant to ampicillin, trimethoprim and vancomycin. Enzymes such as β-lactamases can allow for resistance to penicillin-based antibiotics including ampicillin (Jacoby 2009) and this enzyme appears to be common in *E. coli, K. pneumoniae* and *Pseudomonas* spp. (Jacoby 2009). This could account for the high frequency of ampicillin resistance as *E. coli,* and *Pseudomonas* spp. are common in water and sponges (Cabral 2010; Fu *et al.* 2013; Keller-Costa *et al.* 2014), and all coliform isolates in this study exhibited ampicillin resistance.

The gemmule surface also contained bacteria with resistance to vancomycin and trimethoprim. Coliform isolates in this study were all resistant to vancomycin, but this is

unsurprising, as this antibiotic does not inhibit any Gram-negative bacteria (Harwood *et al.* 2000). Even among Gram-positive bacteria, high resistance to vancomycin particularly from enterococci has been associated with both cattle farms and hospital waste (Daniels 2011; Morris *et al.* 2012). These bacteria can also survive wastewater treatment and are thus discharged into rivers (Morris *et al.* 2012), which could explain why the only *Enterococcus* isolate from the gemmules in this study exhibited vancomycin resistance. enterococci have the ability to pass this resistance on to other bacteria including *S. aureus* (Gardete & Tomasz 2014); hence such a transfer to other bacteria on the gemmules surface is possible, especially if these bacteria become stressed as their host dies.

Generally, reported frequencies of resistance to trimethoprim in bacteria were lower than in this study. Trimethoprim resistant Enterobacteriaceae include *E. coli,* and salmonella but these have the ability to pass on this resistance trait through HGT (Threlfall 2002; Blahna *et al.* 2006; Henriques *et al.* 2006). In just one decade, the relative frequency of multidrug resistant Salmonella isolates from food and water samples with resistance to trimethoprim increased from 0 to 10% in the UK (Threlfall 2002) but at that time the prevalence was still relatively low. Henriques *et al.* (2006) recorded 20% of Enterobacteriaceae with resistance to ampicillin and trimethoprim-sulfamethoxazole from estuaries, but this was far exceeded by the current study with over 95% resistance among isolates from gemmules. The occurrence of high resistance in gemmule bacteria to trimethoprim was unknown, but as trimethoprim can be applied to aquatic animals (Minogue *et al.* 2012) this may provide an explanation. Although there is not a fish hatchery upstream of where the gemmules were collected, some of the lakes are stocked with fish for recreational angling (Mr F. Green 2016 pers. comm 8$^{th}$ September). These fish may have been treated with antibiotics prior to release which could be excreted into the lakes thus exposing bacteria to these substances and leading to a rise in resistance.

In about half of the samples gemmule bacteria were resistant to erythromycin. This frequency of resistance was much lower than that previously reported for the bacteria community in a river of Northern Ireland and Moore *et al.* (2010) found that 97% of culturable bacteria shared this resistance trait. However, the concentration of erythromycin used in the current study was higher, 32 µg ml$^{-1}$ as opposed to 15 µg ml$^{-1}$ used by Moore *et al.* (2010). Erythromycin resistance has been recorded in waterborne enterococci and *Campylobacter* spp. (Arvantidou *et al.* 2001; Moore *et al.* 2001).

Arvantidou *et al.* (2001) recorded erythromycin resistance in 57% of the enterococci samples from coastal water. This level was similar to those recorded from the gemmules, but the gemmules were not associated with high numbers of enterococci. There was a high frequency of erythromycin resistance in bacteria from *E. fluviatilis* gemmules.

The resistance of bacteria from gemmules to rifampicin and tetracycline was low. Moore *et al.* (2010) had not found any culturable aquatic bacteria from a Northern Irish river resistant to rifampicin and thus expectations for this study had been low. Tetracycline resistance of gemmule bacteria in this study was far lower than values reported by Moore *et al.* (2010) who had found that 93% of culturable aquatic bacteria from a Northern Irish river were resistant to this antibiotic despite using higher concentrations of tetracycline on their plates. Typically, bacteria which exhibited tetracycline resistance include campylobacter, enterococci and salmonella (Threlfall 2002; Moore *et al.* 2010; Daniels 2011). This study's single *Enterococcus* isolate showed resistance to all antibiotics tested and further highlighted the problem of a rise in multidrug resistance within this bacterial group (Doud *et al.* 2014). The occurrence of genes controlling efflux pump reactions in enterococci like *E. faecalis* also allows these bacteria to tolerate a range of antibiotics (Lee *et al.* 2003) and this ancient resistance trait (Lupo *et al.* 2012; Mahmood *et al.* 2016) may have helped this bacterium to withstand the test concentrations in this study.

## 6.5 Summary

Disinfection of gemmule surface with hydrogen peroxide was not sufficiently effective at inactivating bacteria from the surface. This means that the origin of bacteria remained uncertain in microcosms with hatched sponges. The gemmule surface contained bacteria which exhibited resistance to ampicillin, erythromycin, rifampicin, tetracycline, trimethoprim and vancomycin. These bacteria included a fluorescent *Pseudomonas* sp. resistant to ampicillin and trimethoprim. The *Pseudomonas* sp. was incorporated into the growing sponges and also released into the water upon hatching offering evidence for bacteria transfer between generations of sponges.

# 7. Antimicrobial effect of freshwater sponge extracts - *Spongilla lacustris* and *Ephydatia* spp.

This chapter aimed to investigate the potential of sponge extracts to act as an antimicrobial agent against bacteria. Methanol extracts of gemmule-grown and river sourced *Ephydatia* spp. and *S. lacustris* were tested against: *Acinetobacter baumanniii*, *E. coli*, *E. faecalis*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Staphylococcus epidermidis*. Specific strains of these bacteria have acquired or developed multiple antibiotic resistance, causing clinical infections that are difficult to treat. They are likely to occur in the aquatic environment where sponges can filter them from the water for food, as shown with *E. coli* and *E. faecalis* in previous chapters, or for symbiosis. Sponges also need defence mechanisms to prevent bacterial infection. The novelty of this experimental chapter was the use of freshwater sponge extract from different sites, and from adult and gemmule-grown sponges to test for inhibition with a variety of bacteria.

## 7.1 Introduction

Sponges are reliant on bacteria as a food source and for symbiosis (Gernert *et al.* 2005). Their tissue can harbour high densities of bacteria, e.g. $1 \times 10^6$ cfu $g^{-1}$ of *Pseudomonas* spp. in *Halichondria panicea* (Müller *et al.*, 1981). These high numbers reflect the abundance of only one bacterial group, but symbiotic bacteria represent a diverse range of species with 32–3000 bacteria species in an individual sponge (Thacker & Freeman 2012). The extent of bacteria diversity in a single sponge is similar to that in water, although the species assemblages may be very different with around 40% of bacteria species in sponges being sponge specific (Thomas *et al.* 2016). All these bacteria have the potential to infect a sponge but pathogenicity appears to vary strongly between individual groups, e.g. while *Vibrio anguillarum* and *Vibrio alginolyticus* infect marine sponges, others including *E. coli* appear to be harmless to them (Fu *et al.* 2013). To protect themselves from bacterial infection, sponges have a basic immune system whereby they contain a wide range of NLR (Nucleotide-binding domain and Leucine-rich repeat containing genes) which allow sponges to detect pathogenic cells before binding to them, thus preventing infection (Böhm *et al.* 2001; Fu *et al.* 2013; Degnan 2015). Sponges can also contain molecules which have antimicrobial effects such as halistanol-trisulphate found in *Petromiea citrina* (Marinho *et al.* 2012).

In experiments with extracts from marine sponges, growth inhibition has been observed for several nosocomial bacteria including: *E. faecium, E. coli, K. pneumoniae* and *S. aureus.* This is evidence for the existence of antimicrobial properties (Marinho *et al.*

2010), which prevent the growth of these bacteria and thus avoid subsequent infection of sponge tissue. However, it remains a challenge to identify the true source of the observed antimicrobial effects, as they could originate from sponges themselves, or from their symbiotic bacteria (Marinho *et al.* 2012; Keller-Costa *et al.* 2014; Eythorsdottir *et al.* 2016). Indeed, bacteria isolated from sponges have shown antimicrobial properties against bacteria and fungi including *E. coli, E. faecalis, Rhizoctonia solani* and *Candida albicans* (Keller-Costa *et al.* 2014; Eythorsdottir *et al.* 2016). Therefore, experimental verification is required, whether antimicrobial effects are exerted by cells of the sponge tissue or its microbial symbionts, which not only include bacteria but also fungi. Some of the difficulties in separating antimicrobial effects between the potential sources are challenges in attaining microbe free sponge tissue and to isolate and grow some of the symbiotic microorganisms without sponges.

The lifecycle of freshwater sponges could help to separate the antimicrobial properties of sponges from their symbiotic bacteria. Freshwater sponges form gemmules during unfavourable conditions, which can be treated to disinfect their external surface (Rasmont 1970). This means that sponges can be hatched in a laboratory setting with minimal numbers of microbes, i.e. they would only be exposed to microbes associated with the gemmules. Sponges collected in the field can hardly be disinfected without simultaneous death of the sponge tissue; therefore, such samples contain a diverse microbial community. A comparison of antimicrobial effects observed in adult sponges collected from natural sites and gemmule-grown sponges reared in the laboratory may therefore offer opportunities to determine the antimicrobial effect from sponges with a full and limited symbiotic community. No other studies testing the difference in antimicrobial effects between adult and gemmule-grown sponges have been found.

Previous studies of the antimicrobial effects of marine sponge extracts showed wide variations in the effectiveness between sponge species (Marinho *et al.* 2010) which has not been tested in freshwater sponges. The different properties of sponge extract could again be due to their microbial community as different sponges have different symbionts (Gernert *et al.* 2005; Costa *et al.* 2013; Eythorsdottir *et al.* 2016). Some of these symbionts are found in a wide number of sponge species while most are more specialist and restricted to one sponge species (Thomas *et al.* 2016). Marine sponges have also been collected from different areas, but it remains to be tested whether this impacts on the effectiveness of the extracts. Hoppers *et al.* (2015) and Eythorsdottir *et al.* (2016) are

among the research groups which combined different sponge species into one sample extract to test antimicrobial properties. Although the mixing of sponges for extracts would enhance the chances of finding an effective antimicrobial solution, it is unclear whether mixing sponges with less effective antimicrobial properties and those showing greater inhibition could reduce the overall efficacy of the extract.

This study tested the inhibitory effect of freshwater sponges (*E. fluviatilis* and *S. lacustris*) on the growth of eight nosocomial bacterial strains. Antimicrobial effects of extracts from river derived adult sponges and laboratory reared gemmule-grown sponges were compared for each species. The impact of site specific factors on the effectiveness of adult sponge extracts was explored further by comparing samples from different river sites and by mixing sponge extracts from two sampling sites.

*Aim and objectives*

The aim of this section was to test if freshwater sponge extracts have an inhibitory effect on the growth of selected nosocomial bacteria. The rationale for the existence of an antimicrobial defence system in sponge colonies was that without an ability to prevent excessive microbial growth in their tissue, sponges would become overgrown and would thus inevitably succumb to microbial infection.

The objectives were:

1. Identification of the existence of antimicrobial effects in extracts of freshwater sponge tissue through growth inhibition tests on selected bacterial strains (H7.1).
2. Comparison of the inhibitory effect of sponge extracts between laboratory-reared sponges grown from gemmules with a low diversity microbiome and adult wild sponges with a high diversity microbiome (H7.2).
3. Comparison of bacterial growth inhibition by sponge extracts to those by an established antimicrobial plant extract and an inorganic chemical substance with antimicrobial properties (H7.3).
4. Comparison of the bacterial growth inhibition by sponge extracts from different rivers and mixtures of these extracts (H7.4).
5. Comparison of the bacterial growth inhibition of sponge extracts from different sites and species in individual rivers (H7.5).

## 7.2 Methods

Three separate trials were carried out with the species *S. lacustris* and *Ephydatia* spp. (Figure 7.1). Trial 1 involved testing laboratory reared sponges grown from gemmules and samples of adult colonies from rivers for antimicrobial properties. Trial 2 compared the antimicrobial properties of adult *S. lacutris* extracts from two sites and of mixtures at different source ratios. Trial 3 collected sponge samples from six sites on two different rivers. The same methods were used for the trials unless otherwise stated using the general method outlined in Figure 7.2.

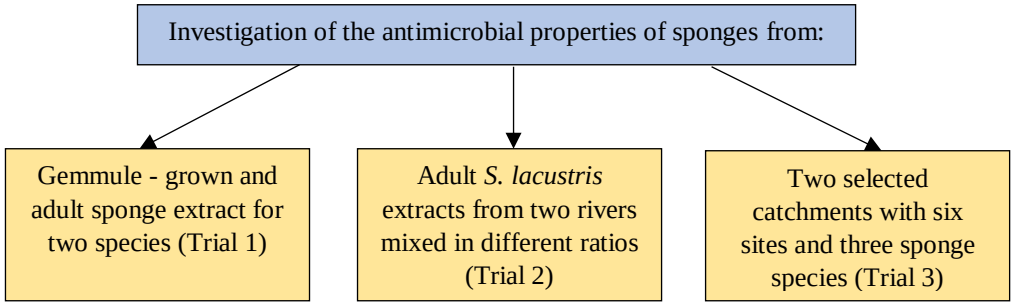



Figure 7.1. Trials to establish the antimicrobial effect of sponges against nosocomial bacteria.

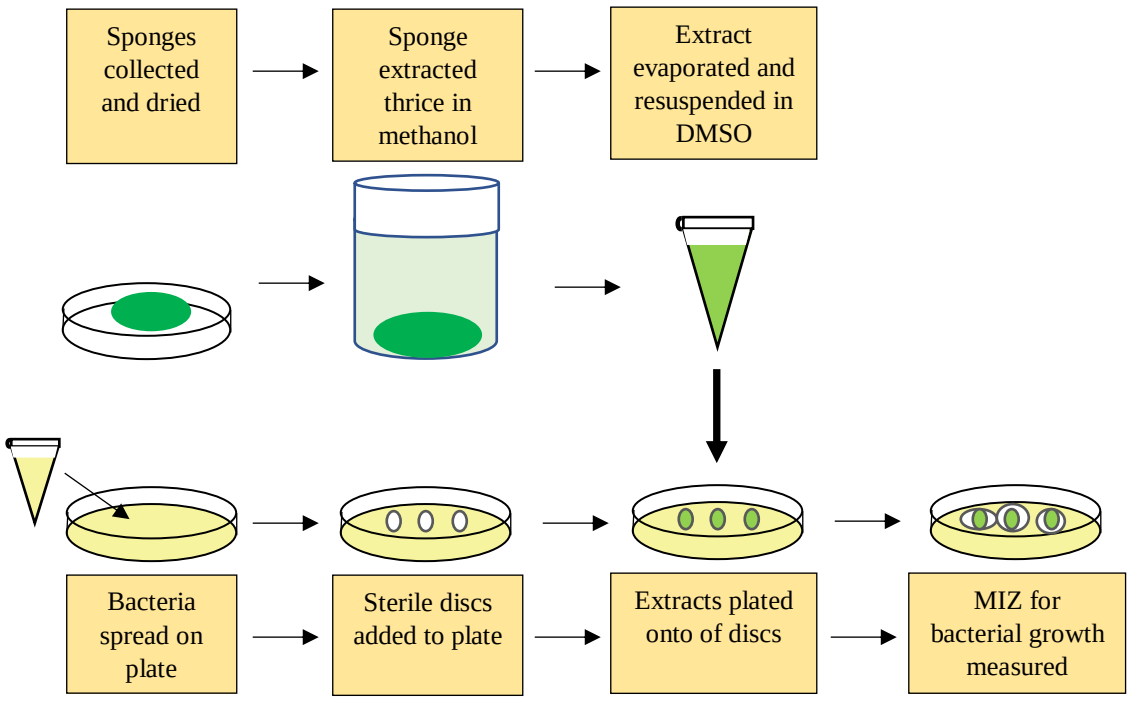
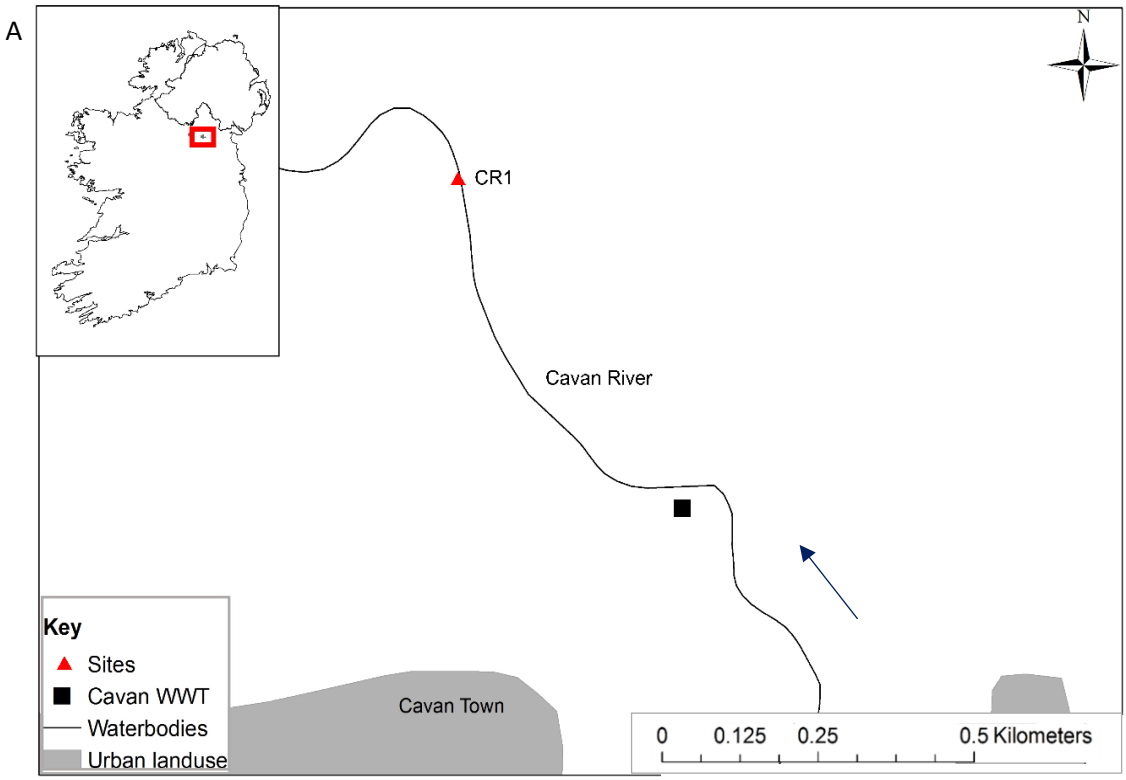



Figure 7.2. Experimental protocol to test the antimicrobial properties of sponge extracts where MIZ = minimal inhibitory zone.

### *Sponge sample collection*

Sponges were collected from three sites (Figure 7.3). Downhill River (Co. Londonderry) has a small rural catchment, Rag River (Co. Cavan) has a larger catchment containing many lakes and rural inputs, and Cavan River (Co. Cavan) has a larger catchment with

rural and urban wastewater inputs. Gemmules from these rivers were collected in the winter and live sponges were collected in the summer. For trial 1: extracts of sponges from laboratory hatched and river sponges were compared for two species of freshwater sponges: *E. fluviatilis* and *S. lacustris*. *E. fluviatilis* was collected from Cavan River, and *S. lacustris* were collected from Rag River. Additional *S. lacustris* gemmules was collected from Downhill River. For trial 2: the same adult *S. lacustris* extract from Rag River was used, and adult *S. lacustris* was collected from Downhill River. For Trial 3: fresh sponges were collected from three sites in Downhill River (DH1-3) and three sites in Rag River (RR1-3). Samples DH1 and DH2 were from the same site, but had been split by sponge species (*S. lacustris* and *E. fluviatilis*) while all other sites contained a single sponge species (Table 7.1). 15 individual sponges were collected from each of these sites.



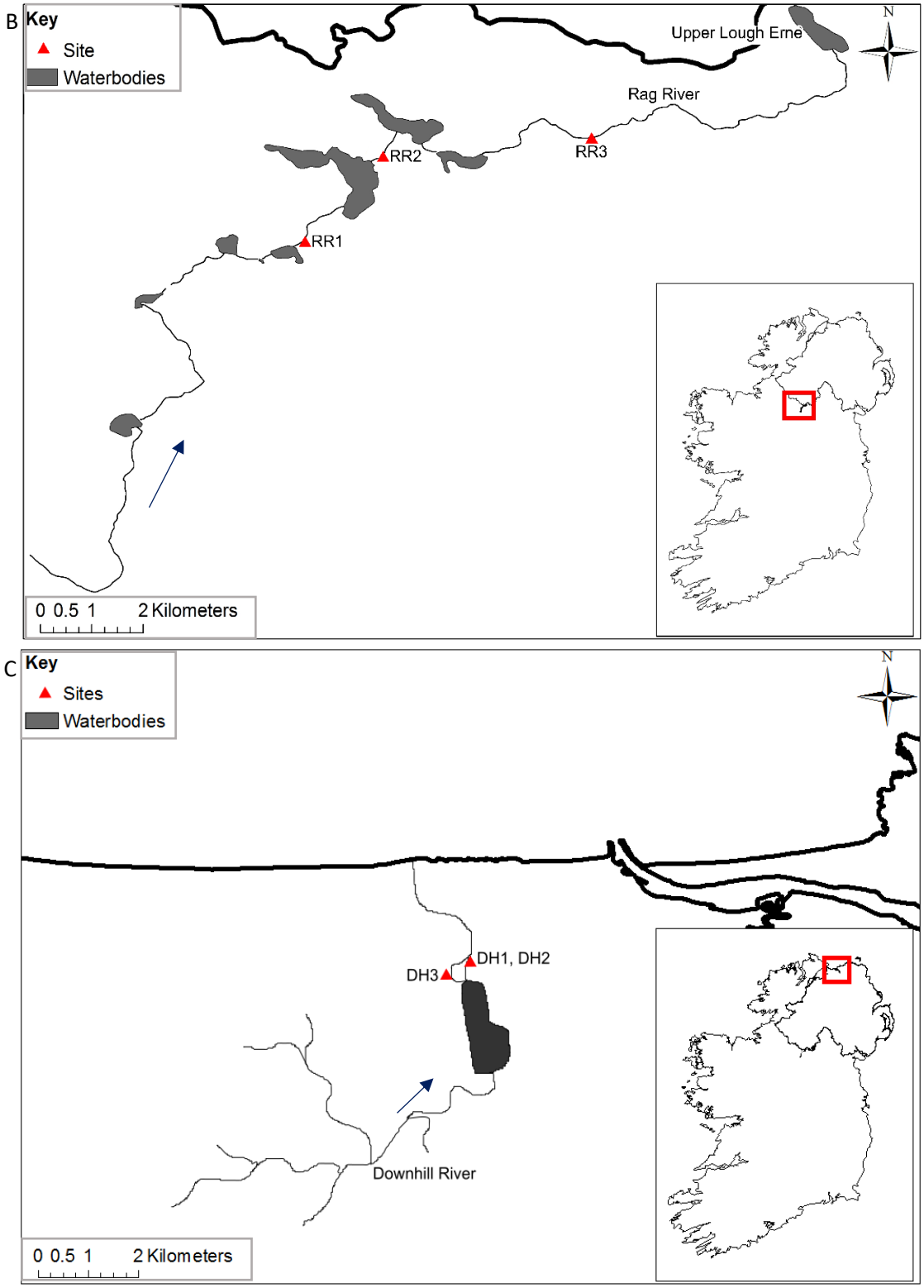



Figure 7.3. Collection sites for sponges and gemmules used to investigate the antimicrobial effects of sponge extracts. A) Cavan River, B) Rag River, C) Downhill River. → = direction of flow

*Abundance of enterococci in sponge tissue*

In trial 3, a subsample was removed from each sampled sponge as a 6 $mm^2$ sponge disc, retrieved with the top of a 1 ml pipette tip. The discs were washed with sterile water before they were cut into small sponge fragments grouped by collection site. The

fragments were added to a universal tube with 10 ml sterile water to form a composite sample from each site. The universal tubes were vortexed for 2 minutes to extract bacteria from the sponges. Each sample was serially diluted tenfold. Six 20 µl drops from each dilution were plated onto Slanetz & Bartley medium. These plates were incubated for 48 h at 37 ℃ for selective growth of *Enterococcus* as an indication of the bacterial abundance in sponge tissue.

*Sample preparation before extraction*

Gemmules (for trial 1) were treated with 1% $H_2O_2$ for 10 min and stored in sterile water at 4 ºC until needed. Gemmules were hatched onto sterile glass petri dishes in 10 ml of UV treated (10 min at 254 nm) mineral water and incubated at 20 ℃. Three weeks after hatching, the water was removed, and the sponges were left to dry (pilot studies had confirmed that in the absence of feed sponges would reach their maximum colony size at this time, see appendix 2). After drying for two weeks, sponges were scraped off the petri dishes and ground into powder using a sterile mortar and pestle. The dry mass was recorded for each species.

Adult sponges (Trials 1, 2 and 3) were collected and placed into a container with river water. In the laboratory each sponge was washed three times with sterile water (ELGA Purelab Ultra grade, autoclaved 121 ℃ for 60 min). The sponges were placed into containers and dried at 20 ℃. Each sample was identified through spicule preparations as described in Section 3.2. After four weeks, the dried sponges were ground into powder as described for the gemmule-grown sponges, and the dry mass was recorded for each sample. In trial 3 before the extracts were ground, a single species composite sample from each site was made with equal weights of 15 dried specimens.

*Aloe vera* leaf extract was used as a positive control. An *Aloe vera* leaf was split so that the gel-filled parenchyma in the centre of the leaf could be removed. This was dried, ground and weighed in the same way as the sponge tissue samples above.

*Methanol extraction of samples*

Methanol was chosen as the solvent for extraction based on Hoppers *et al.* (2015). Otherwise, the method of Pejin *et al.* (2014) was used to produce methanol extracts of each sponge and *Aloe vera*. 4 g dry mass of each sample was placed into 300 ml of methanol (Sigma-Aldrich 99.9%) and left for 1 h (samples were stirred every 15 min to

resuspend settled particles). Each sample was filtered and the solids on the filter scraped into a fresh 300 ml of methanol. This was repeated once more, so that a triple methanol extraction was achieved for each sample. The remaining solids were dried and weighed to determine the extract concentration in the methanol.

The filtrates from the three extractions were combined for each sample and dried with a rotary evaporator (Buchi Rotavapor R-210) at 45 ℃. The dried extract for each sample was resuspended in 5 ml DMSO (Thermo scientific 99.5%) and stored at -20 ℃ until use (Pejin *et al.* 2014). The concentrations of the extracts ranged from 16 mg $ml^{-1}$ to 191 mg $ml^{-1}$ as shown in Table 7.1. In Trial 2, ratios of the Downhill River to Rag River samples were prepared by mixing extracts to give the following proportions (Downhill: Rag River) - Downhill (D100:R0), D75:R25, D50:R50, D25:R75, Rag (D0:R100) with the same overall concentration.

Table 7.1. Concentrations of *Aloe vera* and sponge extracts in each trial. Sites of collection are shown in brackets and relate to Figure 7.2.

| **Trial** | **Extract** | **Concentration (mg $ml^{-1}$)** | **Irish grid reference for collection site** |
|---|---|---|---|
| 1 | *Aloe vera* | 191 | |
| | Gemmule-grown *S. lacustris* (RR2) | 16 | 330300, 217900 |
| | adult *S. lacustris* (RR2) | 51 | 330300, 217900 |
| | Gemmule-grown *E. fluviatilis* (CR1) | 71 | 369000, 241400 |
| | adult *E. fluviatilis* (CR1) | 47 | 369000, 241400 |
| 2 | *Aloe vera* (from trial 1; diluted) | 51 | |
| | adult *S. lacustris* (from trial 1; RR2) | 51 | 317800, 230200 |
| | adult *S. lacustris* (DH1) | 51 | 435400, 275800 |
| 3 | *Aloe vera* (from trial 1) | 191 | |
| | *S. lacustris* (DH1) | 35 | 435400, 275800 |
| | *E. fluviatilis* (DH2) | 41 | 435400, 275800 |
| | *S. lacustris* (DH3) | 28 | 435200, 275900 |
| | *S. lacustris* (RR1) | 27 | 330300, 217900 |
| | *S. lacustris* (RR2) | 35 | 331200, 216800 |
| | *E. muelleri* (RR3) | 26 | 331700, 216200 |

A 14 mM solution of silver nitrate in sterile water was the other positive control for antimicrobial growth inhibition based on Choi *et al.* (2008).

*Bacteria strains and culture method*

Trial 1: Bacteria cultures were prepared from the following strains: *A. baumannii* (DSM30008), *E. coli* (ATCC25922), *E. faecalis* (DSM12956), *K. pneumoniae* (DSM16358), *P. aeruginosa* (DSM3227), *S. aureus* (non-MRSA; DSM20231), *S. aureus* (MRSA; ATCC43300) and *S. epidermidis* (DSM28319). All bacteria except for *E. coli* and *E. faecalis* were grown in nutrient broth (Oxoid). *E. coli* and *E. faecalis* were grown in tryptone soya broth. For Trials 2 and 3, only *A. baumannii, E. coli, E. faecalis,* and *K. pneumoniae* were used as these had been inhibited by sponge extracts in Trial 1.

*Bacterial growth inhibition methods*

Bacteria were spread onto agar plates by adding 100 µl of liquid culture onto tryptone soya agar (*E. coli* and *E. faecalis*) or nutrient agar (all other bacteria strains). The bacteria were allowed to dry into the agar. Six 5 $mm^2$ sterile filter paper discs (Whatman No.1) were placed onto each agar plate. To each of these discs, 6 µl of extract were pipetted to fully cover the disc as described below. Each extract presented in Table 1, DMSO (negative control) and silver nitrate (positive control) were tested in triplicates against the bacterial strains. Plates containing *A. baumannii* were incubated at 30 °C and all other plates at 37 °C. The diameter of zones where bacteria did not grow around the discs were measured after 24 and 48 h to record the MIZ.

*Data visualisation and analysis*

Arithmetic means and standard error values were calculated for each sample. MIZ sizes for the extracts of each bacteria strain were visualised in bar charts. Statistical analysis was completed in SPSS (IBM v22) with separate analysis for each trial. Bacteria that were not inhibited by any sponge extract were removed from statistical analysis. All data were tested for normality using the Kolmogorov–Smirnov test. As there were no normal distributions, the non-parametric Kruskal-Wallis test was applied with a significance level of 0.05. Due to significant differences for all trials, Mann-Whitney U tests with Bonferroni correction were used for pairwise comparisons. The positive controls (silver nitrate and *Aloe vera* extract) were also tested as above. As there was a significant difference between these controls for each bacteria strain, every sponge sample was tested separately against each of the controls. The enterococci from sponges at different sites (Trial 3) were compared using the Kruskal-Wallis test with a 0.05 significance level before Mann-Whitney U Test was used for pairwise comparison as described above.

Extracts from DH1 and DH2 were analysed as one sample, because species identification was completed after the bacteria analysis.

## 7.3 Results

Sponge methanol extracts from various locations, and from gemmule-grown and adult sponges were tested for growth inhibition to selected nosocomial bacteria. The inhibitory effect of sponge extracts was compared to those of *Aloe vera* and silver nitrate. In all trials, none of the bacteria were inhibited by the addition of DMSO to the discs but, all bacteria strains were inhibited by silver nitrate.

*Trial 1 – Antimicrobial properties of adult and gemmule-grown sponges*

None of the sponge extracts inhibited the growth of *P. aeruginosa*, *S. aureus* (non-MRSA) and *S. aureus* (MRSA) and only the *Aloe vera* inhibited *S. epidermidis*. All other bacteria were inhibited by at least one of the sponge extracts (Figure 7.4). MIZ differed significantly between adult *S. lacustris* and all other sponge extracts for *A. baumannii*, *E. coli* and *E. faecalis* ($U=9\text{-}18$, $p<0.001$), because only the adult *S. lacustris* inhibited these bacteria. *K. pneumoniae* was inhibited least by adult *E. fluviatilis* but the other extracts showed similar inhibition. The most inhibited bacteria strain was *K. pneumoniae* which was inhibited by all extracts. The most efficient extract was from adult *S. lacustris* which inhibited half of the strains tested.

Compared to the controls, the MIZ for all bacteria strains were significantly larger in exposure to silver nitrate than for any of the extracts ($U\leq1$, $p<0.001$). Sponge extracts (except for adult *E. fluviatilis*) showed a significantly higher inhibitory effect against the growth of *K. pneumoniae* ($U=244\text{-}295$, $p\leq0.009$) than *Aloe vera* (Table 7.2). The adult *S. lacustris* extract also inhibited the growth of *A. baumannii*, *E. coli* and *E. faecalis* more than *Aloe vera*. The latter did not inhibit *E. coli* and *E. faecalis*, which accounted for these significant differences. *Aloe vera* had a significantly larger inhibition effect on the growth of *S. epidermidis* than all sponge extracts, which showed no effect on this bacteria strain. Growth of *A. baumannii*, was more inhibited by adult *S. lacustris* extract than by *Aloe vera*; extracts from other sponges did not inhibit this bacteria strain at all.

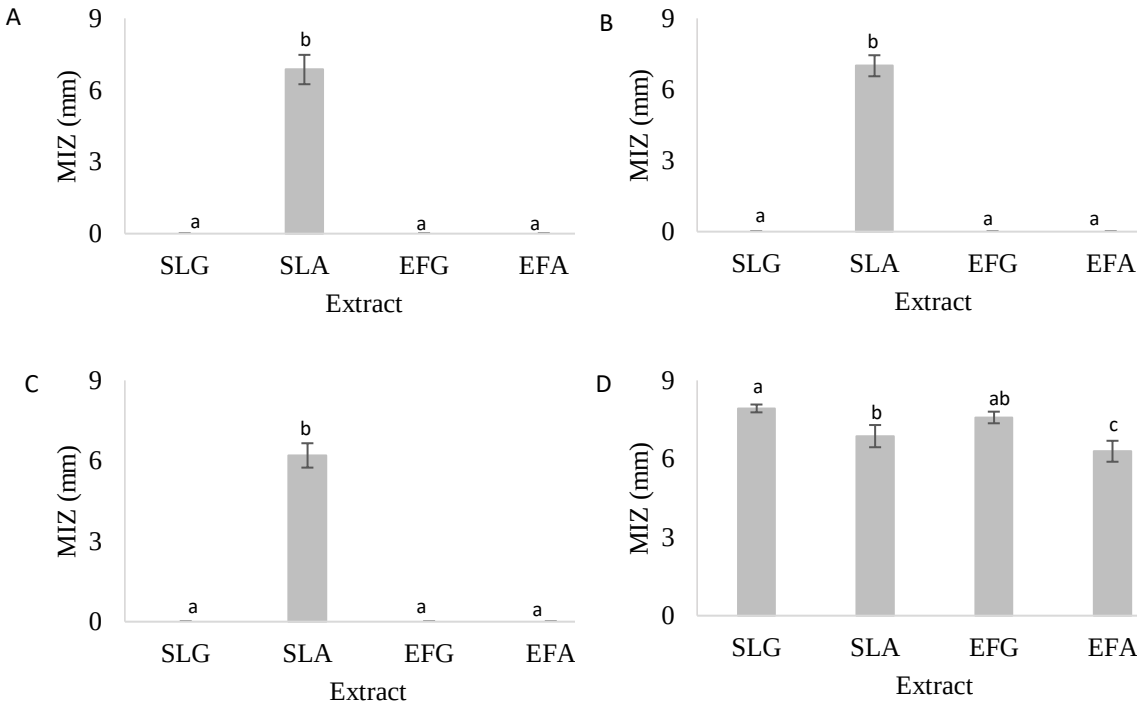



Figure 7.4. Arithmetic means and standard error values for the maximum inhibitory zone (MIZ) of bacterial growth around diffusion discs for gemmule-grown and adult sponges. SLG – *S. lacustris* from gemmule, SLA – adult *S. lacustris*, EFG – *E. fluviatilis* from gemmule and EFA – adult *E. fluviatilis*. A) *A. baumannii*, B) *E. coli*, C) *E. faecalis*, D) *K. pneumoniae*. Different letters represent a significant difference in pairwise comparisons with Mann Whitney U tests ($p<0.05$).

Table 7.2. Mann-Whitney U Test for differences in the minimal inhibitory zone between *Aloe vera* (positive control) and sponge extracts (Trial 1), where AVL – *Aloe vera*, SLG – *S. lacustris* from gemmule, SLA – adult *S. lacustris*, EFG – *E. fluviatilis* from gemmule and EFA – adult *E. fluviatilis*, ** - significant to 0.01 level.

| Samples | *A. baumannii* | *E. coli* | *E. faecalis* | *K. pneumoniae* | *S. epidermidis* |
|---|---|---|---|---|---|
| AVL vs. SLG | <0.001** | | | <0.001** | <0.001** |
| AVL vs. SLA | 0.006** | <0.001** | <0.001** | 0.009** | <0.001** |
| AVL vs. EFG | <0.001** | | | <0.001** | <0.001** |
| AVL vs. EFA | <0.001** | | | 0.252 | <0.001** |

*Trial 2 – Antimicrobial properties of sponge extracts mixed from two sites*

All bacteria strains were inhibited by *Aloe vera,* and the *S. lacustris* extract from Rag River. *K. pneumoniae* and *E. coli* were inhibited by all sponge extracts, but *A. baumannii* and *E. faecalis* were only inhibited when extracts from Rag River sponges contributed at least 50% or 75% to the tested mixtures respectively.

For all tested bacteria strains, the antimicrobial effect of pure extract of Rag River sponges significantly exceeded that of any mixture with extracts of *S. lacustris* from Downhill River (Figure 7.5). *A. baumannii* was only inhibited by samples containing more than 50% of the sponge extract from Rag River, but this only significantly inhibited the growth above 75%. *E. coli* was inhibited by sponge extracts from both rivers, but inhibition was maximal for mixtures containing 75% or more sponge extract from Rag River. The growth of *E. faecalis* was only inhibited by tested extracts with a contribution of Rag River sponges above 50%. *K. pneumoniae* was inhibited by sponge extracts from both rivers, but inhibition was significantly higher with pure Rag River sponge extract and significantly lower with pure Downhill River sponge extract. Mixtures of these extracts did not differ from each other in inhibition strength irrespective of the mixing ratio and their MIZ values covered an intermediate range between those recorded for pure extracts.

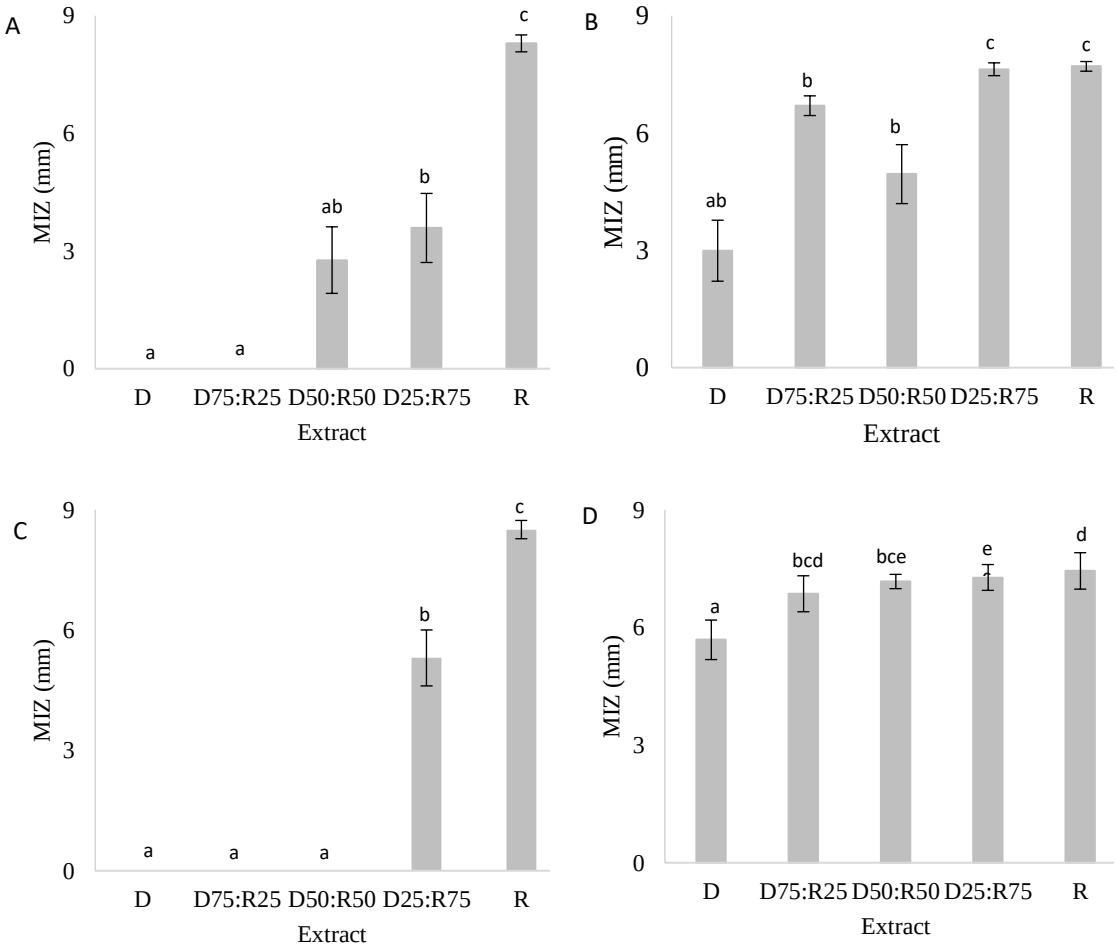



Figure 7.5. Arithmetic means and standard error values for the maximum inhibitory zone (MIZ) of bacterial growth around diffusion discs for mixed sponge extracts. D – Downhill River *S. lacustris* and R – Rag River *S. lacustris*. A) *A. baumannii*, B) *E. coli*, C) *E. faecalis*, D) *K. pneumoniae*. Different letters represent a significant difference in pairwise comparisons with Mann Whitney U tests ($p<0.05$).

In pairwise comparisons of growth inhibition by the positive controls and those by sponge extracts, silver nitrate had a significantly stronger effect than any of the extracts ($U \leq 0.002$, $p<0.001$). Although *Aloe vera* inhibited all bacteria strains in the test, its efficacy varied both with respect to bacteria strains and in comparison with sponge extracts (Table 7.3). *Aloe vera* exerted a significantly higher growth inhibition on *A. baumannii* than any mixture containing sponge extract from Downhill River, and to *E. faecalis* if *S. lacustris* from Downhill River contributed 50% or more to the tested extract. Inhibition of *K. pneumoniae* was generally similar between *Aloe vera* and sponge extracts. *Aloe vera* exerted significantly weaker inhibition effects on *E. coli* and *E. faecalis* than pure sponge extract from Rag River.

Table 7.3. Mann-Whitney U Test for differences in the minimal inhibitory zone between *Aloe vera* (positive control) and the different sponge extracts (Trial 2). AVL – *Aloe vera* leaf, D - Downhill *S. lacustris*, R – Rag River *S. lacustris*, * - significant to 0.05 level, ** - significant to 0.01 level, ↑ - AVL higher inhibition, ↓ - AVL lower inhibition.

| Extract | *A. baumannii* | *E. coli* | *E. faecalis* | *K. pneumoniae* |
|---|---|---|---|---|
| AVL vs. Downhill | <0.001** ↑ | 0.832 | <0.001** ↑ | <0.001** ↑ |
| AVL vs. D75:R25 | <0.001** ↑ | 0.001** ↓ | <0.001** ↑ | 0.059 |
| AVL vs. D50:R50 | <0.001** ↑ | 0.037* ↓ | <0.001** ↑ | 0.079 |
| AVL vs. D25:R75 | <0.001** ↑ | <0.001** ↓ | 0.111 | 0.034* ↑ |
| AVL vs. Rag | 0.963 | <0.001** ↓ | <0.001** ↓ | 0.628 |

*Trial 3 – Antimicrobial properties of sponge extracts from different sites*

None of the sponge extracts inhibited *E. faecalis* and none of the tested bacteria strains were inhibited by all sponge extracts. Only two samples inhibited *E. coli* (DH2 and RR1), but MIZ values were insignificantly small. However, five of the six extracts inhibited *A. baumannii* and *K. pneumoniae*, with sponge extract from site RR1 being most effective (Figure 7.6). This was the same site where the Rag River extract used in trial 1 and 2 was collected, however, it was not as effective as the original extract. Overall, the samples from Rag River and Downhill River were not significantly different in how they inhibited *A. baumannii* ($U=1709$, $p=0.096$), but Rag River extracts showed significantly higher inhibition of *K. pneumoniae* ($U=2005$, $p=0.001$).

In pairwise comparisons of bacterial growth inhibition by positive controls and the sponge extracts, silver nitrate had a significantly stronger effect than any of the extracts ($U<0.000$, $p<0.001$). Although *Aloe vera* had previously inhibited all tested bacterial

strains, it did not inhibit *E. coli* in trial 3 even though the same sample was used. Inhibition of *E. coli* growth by sponge extracts from DD2 and RR1 were not significantly different from *Aloe vera* (U=171, p=0.791). For all other bacteria, *Aloe vera* showed a stronger inhibition effect than any sponge extract. These differences were significant for all samples with *E. faecalis* (U=45, p<0.001), because only *Aloe vera* inhibited this strain. Inhibition by *Aloe vera* was also significantly higher for *A. baumannii* in comparison to sponge extracts from sites DH1, DH2, DH3, RR2 and RR3 and for *K. pneumoniae* compared to sponge extracts from sites DH2, DH3, and RR2 (U≤62, p≤0.001).

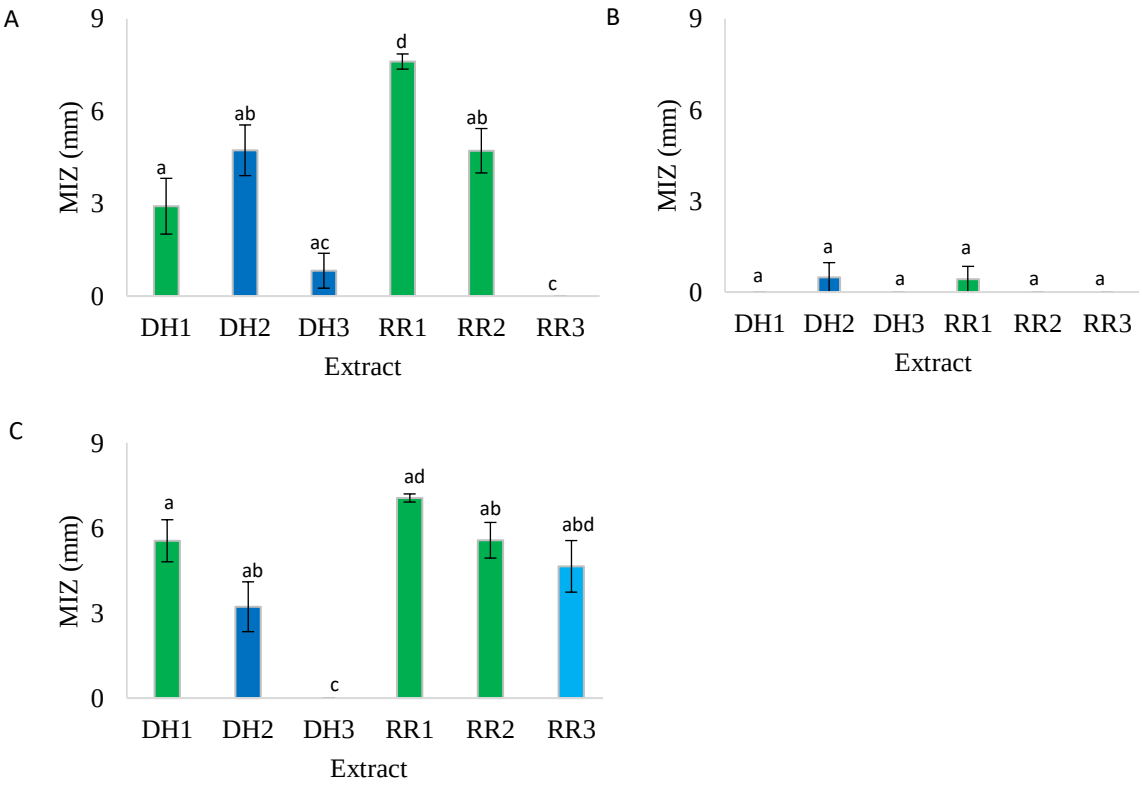



Figure 7.6. Arithmetic means and standard error values for the maximum inhibitory zone (MIZ) of bacterial growth around diffusion discs for sponge extracts from different sites. D – Downhill River and RR – Rag River. - *S. lacustris*, - *E. fluviatilis* and - *E. muelleri*. A) *A. baumannii*, B) *E. coli*, C) *K. pneumoniae*. Different letters represent a significant difference in pairwise comparisons with Mann Whitney U tests (p<0.05).

To identify if greater antimicrobial effects were associated with higher microbial content in the sponges, enterococci in each sample were quantified. Enterococci abundance in sponge tissue was generally higher in sponge samples from Downhill River than in those from Rag River (Figure 7.7). While there was a significant difference between all sites in the Kruskal–Wallis test (H=10.9, p=0.030), there was no significant difference in pairwise comparisons between any two sites.

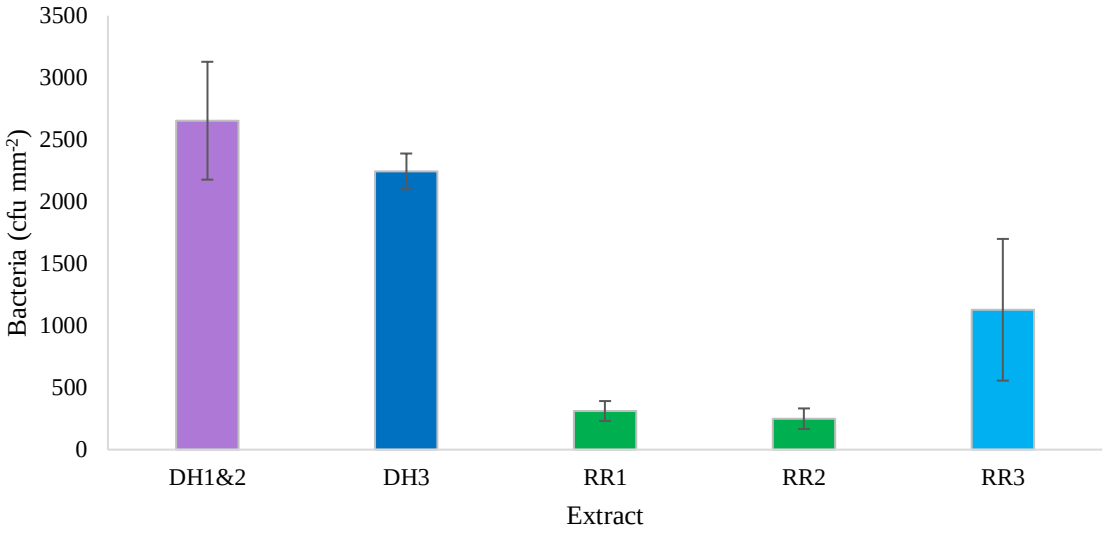



Figure 7.7. Arithmetic means and standard error values for enterococci in sponge samples from different rivers. D – Downhill River and RR – Rag River. - *S. lacustris*, - *E. fluviatilis*, - *E. muelleri* and - *S. lacustris and E. fluviatilis* combined. No significant difference in any pairwise comparisons of sample.

Overall, freshwater sponge extracts had inhibitory effects on the growth of some of the nosocomial bacteria species tested. The main findings were:

1. All freshwater sponge extracts inhibited the growth of *K. pneumoniae*, but extracts from adult *S. lacustris* also inhibited *A. baumannii*, *E. coli*, and *E. faecalis*.
2. Extracts from adult *S. lacustris* showed stronger inhibitory effects than those of gemmule-grown *S. lacustris*, but there was no difference in inhibition effects between adult and gemmule-grown *E. fluviatilis*.
3. In the tested concentrations silver nitrate was a more effective antimicrobial than all sponge extracts, but the latter were more effective than *Aloe vera* in two of the three trials.
4. Inhibition effects by sponge extracts varied between different sample origins from the same river and in composite samples with different mixing ratios.

## 7.4 Discussion

The sponges in this study exhibited antimicrobial effects which can be related to the sponge and their derived microbes. The combined effect can be explained by sponge species and collection site, as the microbial communities of sponges varies with these. The impacts of each factor on antimicrobial properties are discussed below. Inhibitory effects detected in this study are then compared to investigations of other sponges and to

other established antimicrobial solutions. Finally, the potential medical application of sponge extracts will be discussed.

*Contribution of symbiotic bacteria to antimicrobial properties*

Research is now focusing on sponge antimicrobial effects as being from the whole organism including symbiotic bacteria and not just the sponge (Bib *et al.* 2016; Eythirsdottir *et al.* 2016; Saurav *et al.* 2016). In this study extracts from gemmule-grown and adult sponges from natural environments were compared. Growing sponges from gemmules, limited the presence of symbiotic bacteria species in the sponge (see Chapter 6). Thus, laboratory reared sponges with few symbionts could be compared with extracts from river sponges which contained a broad range of symbiotic bacteria. The most effective extracts were from adult sponges, indicating that antimicrobial activities at the very least were enhanced by bacteria in sponges.

Bacteria isolated from sponges which inhibited the growth of other bacteria included: *Bacillus* spp., *Pseudomonas* spp., *Rhodococcus* spp. and *Streptomyces* spp. (Keller-Costa *et al.* 2014; Eythorsdottir *et al.* 2016). *Pseudomonas* spp. isolates from *E. fluviatilis* have also been found to exhibit antimicrobial effects to bacteria and oomycetes potentially through the synthesis of toxic molecules including pyrrolnitrin and hydrogen cyanide (Keller-Costa *et al.* 2014). A fluorescent *Pseudomonas* sp. was found in gemmule grown *E. fluviatilis* (Chapter 6) which may have been present in all sponge extracts used and had a similar effect to the *Pseudomonas* strains isolated by Keller-Costa *et al.* (2014). These bacteria could be responsible for some of the observed inhibitory effects. The taxonomic range of bacteria with antimicrobial effects in sponges may be much wider than the commonly investigated groups of bacteria because investigations on marine sponges suggest a high percentage of bacteria specific to each sponge species (Thomas *et al.* 2016). It is very difficult to isolate the interaction between the sponges and their microbes as few bacterial groups are common in a range of sponges (Thomas *et al.* 2016). Most other bacteria groups were specific to the individual sponge species so their input to the antimicrobial properties of a sponge are more specific. Many of these bacteria could also prove difficult to culture.

Saurav *et al.* (2016) screened extracts from 14 marine sponges for their ability to inhibit the quorum-sensing in bacteria. Quorum-sensing is important in bacterial virulence whereby cell-signalling can cause bacteria to replicate, or release toxic compounds

(Skindersoe *et al.* 2008). After establishing the antimicrobial effect of the sponge/ symbionts, bacteria were then isolated from six sponges and tested for inhibitory effects (Saurav *et al.* 2016). Of 86 tested bacterial isolates, 20% also inhibited quorum sensing (Saurav *et al.* 2016).

*Variation in antimicrobial effect with collection site*

The effectiveness of extracts from the same sponge species varied with collection site which further supported that antimicrobial effects were contributed by the bacterial community, but this could also be linked to variations in the sponges. Sponges from different sites may show, for example, different gene expression due to different immune response which would alter their antimicrobial properties. Both Rag River and Downhill River were exposed to different bacterial regimes which were reflected in sponges (see chapter 4). When enterococci were selected to indicate bacterial abundance, surprisingly their numbers were lower in the sponges with higher antimicrobial effects. Although this does not support the idea that higher bacteria abundance in sponges could cause greater antimicrobial effects, this hypothesis cannot be rejected as enterococci were only one of the many bacterial groups present in the sponges and so total viable bacteria would be a better measure. Thomas *et al.* (2016) recorded different bacteria phyla from sponges with 13-41 phyla in each and so total bacterial counts would be better for the interpretation of whether bacteria abundance affects the antimicrobial properties of sponges.

Mixing of adult *S. lacustris* extracts also caused a reduction in the observed antimicrobial effect. As these extracts were from the same species, they were likely to contain similar symbiotic bacteria groups (Thomas *et al.* 2016), indicating the contribution of location to antimicrobial effects of sponges. However, due to the different bacterial regime in each river, differences in inhibitory effects of sponges could still be due to variation in symbiotic bacteria. Regardless, of the factor causing the observed variation, the better extract became diluted in the other extract thus reducing its inhibitory effect. The most efficient sponge in the current study inhibited half of the bacteria strains tested, however, only a small number of strains were tested. Repeat studies even at the same location did not obtain a second sample with this level of antimicrobial effects further complicating the contributing factor for the antimicrobial effect observed. Therefore, further investigations in to the bacteria in sponges and their antimicrobial effects are required to determine whether the site-specific effect was caused by symbiotic or other bacteria inside the sponge.

The dilution effect observed could also be related to the molecules within the sponge. As sponges contain a complex of their own molecules and those from the symbiotic community, the antimicrobial effect could be from any of these compounds. By containing many molecules, the effect of bioactive compounds could be reduced with dilution in other less active compounds (Marinho *et al.* 2010). This impacts on the antimicrobial effects observed as extracts are a tangle of active and non-active compounds and so the actual concentration of bioactive molecules responsible for the antimicrobial effect cannot be estimated without purification. Bioactive compounds could have affected the tested bacteria strains in several different ways. These include growth inhibition by effect of alteration e.g. membrane degradation, or by interference in signalling processes such as quorum-sensing (Marinho *et al.* 2012; Pejin *et al.* 2014).

In addition to bioactive compounds, it is also possible that sponges contained pollutants from the water which may have enhanced their antimicrobial properties. Marine sponges have been found to retain heavy metals which can affect the growth of natural bacteria, but did not suppress specialised bacteria resistant to elevated heavy metal levels (Hattori 1992; Perez at al. 2004; Drewniak *et al*. 2016). Sediments in freshwater environments can contain elevated levels of atmospherically deposited heavy metals including mercury, lead and nickel, and organic pollutants including polychlorinated biphenyl which could affect all sites (Rippey *et al.* 2008). The pollutants affecting the streams will also be site specific, relating to the complexity of the individual catchments. Rag River had a larger catchment and an abundance of lakes, so concentrations of chemical contaminants could be higher, which may enhance the antimicrobial effect of sponges without negatively affecting the organism (Selvin *et al.* 2009).

*Variation in antimicrobial effect with sponge species*

In this study freshwater sponges were easily cultivated in the laboratory from gemmules with reduced microbial content, from which extracts were produced. The gemmule-grown sponges showed that inhibition of *K. pneumoniae* were likely to be a direct response to the sponges rather than their microbial community as all extracts inhibited this strain. This provided further evidence of a basic immune system within sponges which enables them to inhibit the growth of some bacteria for protection from infection (Böhm *et al.* 2001; Fu *et al.* 2013). There is a need to further understand the ability of sponges to prevent infection. Reports of infections in marine sponges are increasing from unknown

causes (Webster 2007). As these filterfeeders are permanently in contact with microorganisms, they must have defence mechanisms (Böhm *et al.* 2001). Antimicrobial effects are just one of these defence mechanisms. It is also possible that sponges selectively retain bacteria as symbionts if they have antimicrobial effects e.g. *Pseudomonas*. This could be due to the ability of sponges to detect LPS on bacteria and not activate an immune response if the bacteria species is considered beneficial (Gardères *et al.* 2015).

The ability to prevent bacterial growth varied with sponge species. The marine *Petromica citrina* inhibited the growth of 30 different bacterial strains (Marinho *et al.* 2010). However, in the same study other species including *Hymeniacidon heliophile* and *Oceanapia nodose* did not show any inhibitory effect on the growth of any tested bacteria (Marinho *et al.* 2010). This variability could once again be linked to the symbiotic bacteria in the sponge or to the bioactive substances within the sponge, as discussed above. This would impact on the efficacy of each species and the ability to cope with disease. In the current study, the inhibitory effects of adult sponges from *S. lacustris* and *E. fluviatilis* also varied within the same sponge species, so species identity may not be a good indicator of antimicrobial effects.

*Comparative antimicrobial efficacy to other sponges*

The sensitivity of bacteria to sponge extracts varied with bacteria strains. In this study only *K. pneumoniae* was inhibited by all freshwater sponge extracts, but in a test against 12 marine sponges, only one of these species (*Cinachyrella* sp.) inhibited the growth of *K. pneumoniae* (Marinho *et al.* 2010); hence, in comparison to marine sponges the species in freshwater environments appear to show greater inhibitory effects against this potential bacterial pathogen.

Adult *S. lacustris* also inhibited *A. baumannii*, *E. coli*, and *E. faecalis*. When compared to the efficacy of other sponge extracts, *E. coli* was inhibited by 5 of 29 tested sponge species (Marinho *et al.* 2010; Govinden-Soulange *et al.* 2014; Hoppers *et al.* 2015; Saurav *et al.* 2016). *E. faecalis* has not been as widely tested with sponge extracts, but it was inhibited by sponges including: *Biemna tubulosa*, *Haliclona* sp., and *Stylissa* sp. (Govinden-Soulange *et al.* 2014; Hoppers *et al.* 2015). No studies with tests of sponge extracts against *A. baumannii,* a recently emerged multi - antibiotic resistant bacteria were found (Howard *et al.* 2012).

Sponges in this study did not inhibit *P. aeruginosa*, *S. aureus*, and *S. epidermidis*, but other studies have found sponge extracts which could inhibit these bacteria. The ability of sponge extracts to inhibit the growth of *P. aeruginosa*, for example, varied greatly between studies. Similar to this study, no sponges which inhibited the growth of *P. aeruginosa* were found by Marinho *et al.* (2010) or Saurav *et al.* (2016) with 26 different marine sponges in total, tested against this bacteria species. However, the quorum sensing in *P. aeruginosa* was inhibited by the freshwater sponge *Ochridaspongia rotunda* and the marine sponges *Suberites clavatus* and *Ircinia variabilis* which reduced its ability to form biofilms by limiting, for example, pyocyanin production, one of the toxins produced by these bacteria (Pejin *et al.* 2014; Saurav *et al.* 2016). This is further testament to different sponge species having different abilities to inhibit bacterial growth. There is also a rationale for the sponge immune response to vary between locations. For example, if one bacteria species is common in a waterbody and has the potential to infect the sponge, an antimicrobial immune response towards this species is more likely to evolve. Therefore, the antimicrobial effects of sponges could also be more related to sampling site than taxonomic identity.

*Comparative antimicrobial efficacy to other antimicrobial solutions*

Apart from extracts of sponges, microbial growth can also be inhibited by plant extracts including *Aloe vera* or by bacteriotoxic chemicals such as silver nitrate. *Aloe vera* has been widely used in antimicrobial applications to inhibit Gram-positive and Gram-negative bacteria and its antimicrobial properties are well-known ( Lawrence *et al.* 2009; Banu *et al.* 2012), which makes it a useful reference substance for comparative studies. Silver nitrate, as the second reference substance, is an inorganic chemical with very strong antimicrobial properties, and resistance to it is unlikely (Mosselhy *et al.* 2015). Its antimicrobial properties have been attributed to the silver ion, which alters the cell membrane in bacteria, thus reduces the cells' ability to grow and eventually causes death (Jung *et al.* 2008).

The antimicrobial effects of *Aloe vera* varied between individual trials, despite the same extract solution being used throughout. In trial 1 *Aloe vera* did not inhibit the growth of *E. coli* or *E. faecalis* which were however, both inhibited in trial 2. In trial 3, the extract did not inhibit *E.* coli. The reason for the change in efficacy is unknown, especially as the sample was diluted from 191 mg $ml^{-1}$ to 51 mg $ml^{-1}$, in trial 2 where it was most effective.

The same bacteria strains were used throughout, but there may have been a difference in the gene expression of these bacteria at the time of testing. Bhardwaj *et al.* (2012) reported 79% efficacy of *Aloe vera* against *E. faecalis* indicating variation even within the same extract, or different bacterial response depending on cell condition, e.g. in different growth phases. *Aloe vera* has been found to inhibit the growth of *Bacillus cereus, E. coli, K. pneumoniae, P. aeruginosa, Salmonella typhi, S. aureus* and *Streptococcus pyogenes* (Lawrence *et al.* 2009; Banu *et al.* 2012), but in this study it did not inhibit *P. aeruginosa,* and *S. aureus*, possibly due to different methods of extraction being used or differences in the tested bacteria strains. It is also possible as with the sponges that bioactive molecules were diluted with other molecules from the leaf extract.

Silver nitrate, however, was a very effective antimicrobial, preventing the growth of all bacteria. The concentration of silver nitrate which prevented the growth of all bacteria in this study was also effective against autotrophic bacteria communities in wastewater sludge and *E. coli* (Choi *et al.* 2008). In order to take advantage of this antimicrobial effect, manufacturers have started to incorporate silver nanoparticles into surgical masks and medical gowns in attempts to reduce the potential of transferring nosocomial infections in hospitals (Li *et al.* 2006; Mosselhy *et al.* 2015). This was the only antimicrobial solution used where the concentration of the compound responsible for the activity was known. If molecules exhibiting the antimicrobial effect from the sponges and *Aloe vera* were isolated and used in a known concentration, they may exhibit similar strong inhibitory effects.

*Sponges as sources of new antimicrobials for medical applications*

The interest in the antimicrobial properties of sponges and their symbionts is an emerging research topic because in clinical environments the growth of infectious bacteria needs to be regulated by antimicrobial solutions (Zhang *et al.* 2009). Widespread use of antimicrobial and antibiotic compounds has meant that resistance to these chemicals has occurred and so their efficacy are declining (Davies & Davies 2010; Marti *et al.* 2014). Without the discovery of new antimicrobials, humans could be entering a post-antibiotic era where once again there will be deaths from common bacterial infections (Berendonk *et al.* 2015; Kenny *et al.* 2015). Bacteria such as *A. baumannii, E. coli, Enterococcus* sp., *K. pneumoniae, P. aeruginosa, S. aureus* (including MRSA strains) and *S. epidermidis* have become common bacterial pathogens which have multiple resistance to antibiotics (Lyczak *et al.* 2000; Heijnen & Medema 2006; Maragakis & Perl 2008; Patel *et al.* 2008).

Most of these bacteria occur naturally and can even comprise part of the natural human microbiome, but pose a threat in certain situations, particularly to immune suppressed patients after surgery. They can exhibit virulence and cause infections which are difficult to treat due to their antibiotic resistance (Maragakis & Perl 2008; Novais *et al.* 2013).

Therefore, the focus has been placed on finding antimicrobial compounds within organisms including sponges (Govinden-Soulange *et al.* 2014; Hoppers *et al.* 2015). Only *K. pneumoniae* was widely inhibited by sponges in this study. This genus of bacteria is of current concern as there is a rise in hospital spread infections and it often exhibits multidrug resistance (Patel *et al.* 2008). Carbapenem resistance in particular causes high mortality with up to 86% of patients dying (Cheepurupalli *et al.* 2017). In 2016, a woman died after contracting *Klebsiella* sp. resistant to all known antibiotics which prevented treatment (Chen *et al.* 2017). This means that sponge extracts could be further tested for control of this clinically relevant species of bacteria. The next stage of testing for the sponge extracts against *Klebsiella* sp. would involve fractioning the extract to find the bioactive molecule. The molecule causing the antimicrobial effect could be separated by e.g. chromatography (Hoppers *et al.* 2015) and tested for inhibition to establishing the minimal inhibitory concentration (MIC). The MIC can be found for the fraction by testing a range of different concentrations on the same bacteria strain (Marinho *et al.* 2010; Govinden-Soulange *et al.* 2014; Hoppers *et al.* 2015).

## 7.5 Summary

Extracts from freshwater sponges *S. lacustris* and *E. fluviatilis* acted as an antimicrobial growth suppressor of *K. pneumoniae*. Extracts from adult *S. lacustris* also inhibited *A. baumannii*, *E. coli*, and *E. faecalis*, potentially due to the microbial community within this sponge, which was not found in the gemmule-grown sponges. Antimicrobial effects of an extract varied not only with sponge species but also with the site of origin for the same species. Site of origin appeared to be more important than sponge species in determining the strength of antimicrobial effects. Freshwater sponges inhibited growth of bacteria more strongly than *Aloe vera*, but none of these extracts were pure. Therefore, the silver nitrate was the most effective antimicrobial solution but purification of the bioactive compounds from sponges could prove to be as effective.

# 8. General discussion

Infection from ARB is a common health risk in modern society and there is evidence that we may be on the edge of a post-antibiotic era where once again, bacterial infections will not be controlled and people will die from common infections (Kenny *et al.* 2015). Selected strains of *E. coli* and *E. faecalis* are among the pathogenic bacteria, which exhibit multidrug resistance (Arias & Murry 2012; Yang *et al.* 2017). Bacteria can acquire multidrug resistance by a number of mechanisms including transduction and conjugal transfer and evidence for these gene transfers can be found in bacteria isolates (Parsley *et al.* 2010). Evidence of transduction in *E. coli* isolated from activated sludge has been observed from the presence of phage genome which resulted in antibiotic resistance (Parsley *et al.* 2010). However, conjugal transfer is assumed to play a larger role in antibiotic resistance transfer which can occur in isolates of *E. coli* and *E. faecalis* (Phornphisutthimus *et al.* 2007; Conwell *et al.* 2017). The conjugal transfer process relies on a connection between bacteria through which genes can pass (Wilson *et al.* 2010). Once bacteria acquire antibiotic resistance in a host organism, natural environment or clinical setting, they can enter the water from sewage and farm runoff or ineffective WWTP and septic tanks (Baudart *et al.* 2006; Ahmed *et al.* 2005).

Selected bacteria can cause diseases in both animals and humans e.g. *E. coli* and *Vibrio shilonii* (Li *et al.* 2017; Yang *et al.* 2017). In recent years diseases in aquatic organisms has been related to factors including anthropogenic alteration to climate and water pollution (Webster 2007; Webster & Taylor 2012). The infection of aquatic organisms has direct impacts on ecosystem health and the aquatic food web where organisms lose their home and food source as it disintegrates from disease (Webster 2007). As filterfeeding organisms draw water into their bodies, they encounter high abundances of bacteria and so they are exposed to potential pathogens. Therefore, sponges, for example, require defences against disease so they exhibit a basic immune response (Böhm *et al.* 2001; Fu *et al.* 2013) and are also known to contain bacteria with antimicrobial effects (Keller-Costa *et al.* 2014). Their immune response involves 14-3-3 proteins, which are released after infection with e.g. *Vibrio* sp. (Fu *et al.* 2013). In addition, some of the symbiotic *Pseudomonas* sp. in sponges show the potential to inhibit microbial growth to species including *Rhizoctonia solani* and *Bacillus subtilis* (Keller-Costa *et al.* 2014).

There is evidence to suggest that bacteria from water can infect humans, if this water is consumed or used for recreational activities (Solomon *et al.* 2002; Soller *et al.* 2010). These studies include the contamination of food crops with *E. coli* O157: H7 from

irrigation water which entered the food web causing infection (Solomon *et al.* 2002). To monitor water for the potential presence of human pathogens, indicator bacteria such as *E. coli* and *Enterococcus* spp. are used in bathing and drinking water (Ferguson *et al.* 2012; Wiedenmann *et al.* 2006) Conventional sampling for water chemistry which can also be applied for microbial parameters bases decisions on a spot sample of water taken at one point in time (Kirchner *et al.* 2004; Briciu-Burghina *et al.* 2014). This has implications for human health as episodic pollution events may be missed. This means time-integrating sampling methods and rapid quantitative analysis techniques are needed. For example, qPCR has potential to rapidly detect and quantify indicator organisms in the water and whether they originate from human faeces maximising the potential to indicate the presence of pathogenic bacteria (Noble *et al.* 2003; Harwood *et al.* 2014).

The information in the preceding chapters provided insight into how sponges interact with bacteria and can be linked by five main themes divided into three sections: ARB, infection/defence, and interactions with/detection of aquatic bacteria. Each of these will be discussed.

## 8.1 Antibiotic resistance in the environment and gene transfer in association with filterfeeders

Antibiotic resistance is a problem within a clinical setting where it causes mortality and morbidity (Emaneini *et al.* 2016). Among the pathogenic bacteria which exhibit multidrug resistance are enterococci and *E. coli* (Yang *et al.* 2017; Zheng *et al.* 2017). *E. faecalis* and *E. faecium* accounted for 90% of hospital admissions from enterococci infections (Zheng *et al.* 2017). This group started to emerge as a pathogen in the 1970s (Jett *et al.* 1994). One of the most substantial changes in its resistance profile was the development of vancomycin resistance. VRE were first reported in 1986 and rapidly spread from the UK to other countries, including Iran where it was introduced in 2004 (Emaneini *et al.* 2016). The spread of VRE in the UK has been controlled with prevalence of around 10% maintained from the 1990s to the early 2000s (Reacher 2000; Brown *et al.* 2008; Emaneini *et al.* 2016). However, infection numbers from these bacteria are rising around the world and may not be as effectively controlled, thus making VRE a global health concern (Emaneini *et al.* 2016).

ARB are not limited to clinical environments but also occur in aquatic environments, where these bacteria can also be retained in other biological organisms, including

sponges. Selvin *et al.* (2009), for example, found ARB including *Micromonospora, Saccharomonnospora, Vibrio* and *Pseudomonas* in the marine sponge *Fasciospongia cavernosa*. These bacteria and others isolated from the sponge were tested for their resistance to 24 antibiotics including ampicillin, rifampicin and sulphadiazine and exhibited relative resistance frequencies between 48% and 64% (Selvin *et al.* 2009). Beyond sponges ARB have also been found in other aquatic organisms including daphnia and urchins (Eckert *et al.* 2016; González-Aravena *et al.* 2016). The ARB found in the urchins included 42 isolates of Proteobacteria and Actinomycetes with 18 of these isolates exhibiting antibiotic resistance (González-Aravena *et al.* 2016). Eleven of these isolates were resistant to cefotaxime. The urchins sampled in their study were from Antarctica which is a continent with a virtual absence of direct human exposure. This provides an example for either the natural occurrence of ARB in marine environments or the transfer of human-induced ARB through water currents from other areas of the globe. As prey for consumers at higher trophic levels these urchins may also provide a reservoir for the vertical transfer of ARB through the marine food web. In freshwater environments there is evidence of the filterfeeding planktonic daphnia being an important prey for fish (Merle 1967). Daphnia as filterfeeders of algae and bacteria, are also likely to ingest ARB with their food (Eckert *et al.* 2016). The daphnia in experiments by Eckert *et al.* (2016) retained the resistance gene *tet(A)* within their gastrointestinal tract, where this genetic trait can potentially be transferred to their symbiotic bacteria. Theoretically organisms like daphnia or urchins may spread ARB through defecation or by falling victim to their predators.

Once ARB are combined, they can acquire new resistance genes mainly through conjugal transfer once the cells are in contact. Under laboratory conditions, conjugative vancomycin transfer between *E. faecalis* can achieve an efficiency of $10^{-3}$ (Conwell *et al.* 2017). This same type of transfer presumably occurred with sponges in the current study. Evidence for enterococci conjugal resistance transfer has also been reported from studies focusing on the gastrointestinal tract of houseflies and mice. The transfer of tetracycline resistance between *E. faecalis* occurred in flies with a maximal efficiency of $10^{-3}$, while the transfer of erythromycin resistance between *E. faecium* in mice resulted in high faecal transconjugant numbers with up to $10^{6}$ cfu $g^{-1}$ (Lester *et al.* 2004; Akhtar *et al.* 2009). The plasmid responsible for this transfer for vancomycin resistance in Conwell *et al.* (2017) and the tetracycline resistance in Akhtar *et al.* (2009) was pCF10. Therefore, the transfer of a plasmid between bacteria can result in the acquisition of resistance to a variety of

antibiotics and sometimes even contain multiple resistance (Daniels *et al.* 2011). This has implications for the environments whereby ARB can potentially pass on resistance to pathogenic bacteria that could cause clinical infection (Lupo *et al.* 2012; Berendonk *et al.* 2015).

Although conjugal transfer had not been previously demonstrated with sponges before this study, there is potential evidence of this mechanism in the sponge microbiome as isolated bacteria from many sponges including *Scopalina* sp. contained conjugal gene elements including COG3451 (Fan *et al.* 2012). Conjugal transfer is not the only mechanism for the transfer of antibiotic genes as both transformation and transduction occur in aquatic ecosystems (Lupo *et al.* 2012). Transduction is considered important for the transfer of antibiotic resistance in freshwater environments and genome sequencing results from bacterial isolates of marine sponges provide evidence for its occurrence in the sea (Lupo *et al.* 2012; Webster & Thomas 2016). Stress-resistance genes like COG0687 and COG0642 get frequently transferred by transduction and have been found in bacteria isolated from marine sponges (Fan *et al.* 2012; Burgsdorf *et al.* 2015). It is also likely that transformation can occur within sponges. Fan *et al.* (2012) found a COG0758 gene inside sponge bacteria which was associated with this transfer mechanism, but with all the genes outlined above, it was unclear whether their transfer took place in the water environment or sponge. These antibiotic bacteria with acquired resistance can be found in the environment where subsequent gene transfer can occur unchecked, especially as sponges retain around 76% of the filtered bacteria (Rieswig 1975). Therefore, it is also possible for sponges to release bacteria that have acquired resistance in their tissue back into the environment.

To avoid or at least reduce the anthropogenic spread of ARB and their associated genes in aquatic environments, these bacteria and genes should be removed before water is discharged into rivers. Although WWTPs have efficient mechanisms to retain pollutants like nutrients and particulates, even among the most advanced treatment plants many are not specifically equipped to inactivate bacteria e.g. with UV or to retain trace organics such as antibiotics (Baudart *et al.* 2000; Hübner & Jekel 2013; Rajasulochana & Preethy 2016). This means that WWTPs themselves are an environment which may facilitate the transfer of antibiotic resistance. Wastewater and sludge contain high numbers of bacteria, among them e.g. vancomycin resistant enterococci. Oravcova *et al.* (2017) found *vanA* genes mainly from *E. faecium* in 86% of 37 wastewater effluent samples from a WWTP

with secondary treatment. Wastewater in treatment plants often contains unmetabolized antibiotics excreted by patients and antibiotic compounds in the industrial wastewater from pharmaceutical production sites (Sidrach-Cardona *et al.* 2014). Zhang *et al.* (2017) monitored the removal of 31 antibiotics in 12 WWTPs. The most frequently detected classes of antibiotics in the water included fluoroquinolones and sulphonamides. The antibiotic removal efficiency of treatment depended on the antibiotic substance and the individual treatment plant, with one WWTP removing only 21% of the antibiotic sulfapyridine while another site removed 100% (Zhang *et al.* 2017). The removal of sulfapyridine was the lowest recorded removal for any of the antibiotics, while some antibiotics including doxycycline and rifampicin were completely removed by all tested WWTPs (Zhang *et al.* 2017). Generally, WWTPs released some antibiotics, ARB and their genes into the environment; Subirats *et al.* (2017) found a concentration of 18 and 54 ng $l^{-1}$ of ofloxacin, for example, in river water and treated wastewater respectively. They also detected elevated numbers of ARG including *sul1, intl1,* and *ermB* in bacterial biofilm downstream of a WWTP, but the abundance of these genes was site-specific. In comparison to upstream sites one of the two rivers in their study had significantly higher abundance of the s*ul1* gene in biofilm samples from downstream of a discharge point for wastewater effluent (Subirats *et al.* 2017). The other river had significantly higher abundances of *intl1, sul2, sul2, ermB, qnrS, tetM* and *tetW* genes downstream of the discharge point (Subirats *et al.* 2017). The increased detection frequency of ARB and resistance genes near WWTP effluent pipes suggests that such pollution sources could increase the potential for HGT of antibiotic resistance.

The examples highlighted above only focused on the contribution of wastewater towards aquatic ARB and their resistance genes. However, as Ibekwe *et al.* (2011) found, WWTP are not the only source of bacteria. Their counts of *E.* coli, coliforms, enterococci and total bacteria were higher in an urban river than in effluents from two WWTPs. The sources for the faecal pollution in their river were from urban and agricultural runoff. The investigations of ARB with WWTP may reflect the ease of quantifying loads from point source pollution as outlined with nutrients, diffuse sources are hard to quantify (Neal & Heathwaite 2005). The locations at which water was collected for faecal indicator bacteria has also proved to be an important factor in determining bacterial abundance which indicates that bacterial loading regimes are site and river-specific, so no general rule can be applied (Ibekwe *et al.* 2011).

8.2 Sponge infection and defence against disease

According to Degnan (2015) the immune system of sponges is of equal level to those exhibited in vertebrates but the sponge immunity is mainly based on a less complex system including NLR genes. The NLR genes allow the sponge to differentiate between infectious and harmless microbes before binding to harmful cells (Degnan 2015). Sponges can detect, and separate bacterial groups using pattern recognising receptors which bind to bacterial ligands (Degnan 2015). The identification of harmful bacteria thus results in an immune response to protect the sponge. The marine sponge *Amphimedon queenslandica* contains at least 135 NLR genes from the *AqNLR* group (Degnan 2015). In addition to the use of NLR to detect foreign microbes, sponges can also use bacterial LPS to differentiate between bacterial cells. Gardères *et al.* (2015) found that the marine sponge *S. domuncula* detected specific LPS from *Endozoicomonas* and *Pseudoalteromonas*. The detection of LPS can result in the expression of macrophage genes in sponges, but in the study by Gardères *et al.* (2015), the macrophage genes were only released as a response to *E. coli* and not to *Endozoicomonas* and *Pseudoalteromonas* with a 1.8 fold mRNA increase with *E. coli* and a 1 fold increase with the other bacteria species. This shows the ability of sponges to establish different immune responses based on the specific bacteria they are exposed to.

Despite a basic immune system, sponges can succumb to bacterial infection as their cells become overrun with bacteria causing death to the organism (Böhm *et al.* 2001; Fu *et al.* 2013). Among the known pathogens which infect marine sponges like *Rhopaloeides adorabile* is the α – Proteobacterium NW4327 (Mukherjee *et al.* 2009).This bacterium was found to contain an enzyme which digests the sponge's collagen structure thus causing its demise and break-down (Mukherjee *et al.* 2009). Disease in sponges can be recognised by monitoring 14-3-3 genes, which can indicate infection and subsequent immune response (Fu *et al.* 2013). That research group documented the infection of the marine sponge *Hymeniacidon perleve* with *Vibrio* spp. through the aforementioned genetic markers where a reduction in this gene expression was symptomatic of disease. The expression of 14-3-3 gene also reduced further with increasing loads of *Vibrio* from mRNA expression of 0.17 with $3.6 \times 10^4$ cfu $ml^{-1}$ to 0.08 with $3.6 \times 10^4$ cfu $ml^{-1}$ exposure over 6 h. However, they found that *E. coli* did not infect sponges resulting in similar expression of 14-3-3 to those of control sponges without bacteria with mRNA expression of 0.20 for both (Fu *et al.* 2013).

Stress factors including rising temperature and eutrophication appear to have a role in sponge infection, as this can reduce the host's immune system (Webster 2007; Kaluzhnaya & Itskovich 2015). The role of stress factors in disease has not been widely studied in sponges, but different infection systems have been identified in other organisms, e.g. corals. Coral infection is related to the release of a molecule, dimethylsufoniopropionate (DMSP) under stress conditions including elevated temperatures (Li *et al.* 2017). The DMSP molecule is thought to attract *Vibrio* to the coral, which subsequently infects the organism. Li *et al.* (2017) found that the DMSP acted in a comparable manner to the AI-2 quorum sensing molecule released by *Vibrio shilonii* and *Rothia*, thus altering the bacterial community in the coral as a response to stress. These changes in the symbiotic community could cause disease in the host. The strong relationship between bacteria and sponges could facilitate a similar infection model to that observed in coral.

Generally, no disease has been reported for freshwater sponges, however, Kaluzhnaya & Itskovich (2015) did observe bleaching in the freshwater sponge *Lubomirshkia baicalensis* as the sponges lost their symbiotic algae. Although the cause of the bleaching remained unknown, it was attributed to pollution, temperature or eutrophication, which can all trigger disease in a sponge causing the symbiotic bacteria to be exiled. The absence of reports on diseases in freshwater sponges does not mean that freshwater sponges are not affected by diseases, but may instead reflect a sampling bias, since reef systems, for example, are monitored more often than sponges in rivers.

In addition to the type of immune response discussed above, sponges can also produce or acquire molecules which offer them defence against infection. Many sponges have shown antimicrobial effects within a laboratory setting (Marinho *et al.* 2010; Govinden-Soulange *et al.* 2014; Hoppers *et al.* 2015; Saurav *et al.* 2016). One of the more effective sponges was *P. citrina* which inhibited 30 out of 44 bacterial species tested (Marinho *et al.* 2010). This antimicrobial effect was later attributed to the molecule halistanol-trisulphate which damaged cell membranes before causing full lysis and death of the bacterium (Marinho *et al.* 2012). Sponges can also produce molecules, which inhibit the production of biofilm in bacteria. For example, the freshwater species *Ochridaspongia rotunda* inhibited the growth of *P. aeruginosa* biofilm production through the release of the anti-quorum sensing molecule pyocyanin (Pejin *et al.* 2014). This indicates that the sponges themselves can produce chemical defences against bacteria.

Sponges can also derive disease-prevention mechanisms from their symbiotic bacteria. *E. fluviatilis* harbours high abundances of fluorescent *Pseudomonas* bacteria, which exhibit an ability to prevent the growth of other microbes (Keller-Costa *et al.* 2013). Half of the 90 *Pseudomonas* strains isolated inhibited bacterial growth, 35% inhibited protozoan and 32% inhibited oomycetes potentially from the synthesis of molecules including pyoluteorin and hydrogen cyanide (Keller-Costa *et al.* 2013). Antimicrobial *Pseudomonas* sp. have also been isolated from soil which can produce phenazine that prevents fungal growth (Tupe *et al.* 2015). The antimicrobial properties of such bacteria enable their existence in many environments and have even been detected in cores of ancient ice which has formed more than 750, 000 years ago (Christner *et al.* 2003) and is thus testament to the adaptation and wide ranging existence of this genus. This may also explain the strong relationship between sponges and these bacteria which have evolved together over hundred thousands of years whereby they coexist even in laboratory sponges as demonstrated with *S. domuncula* and *Pseudomonas* (Böhm *et al.* 2001).

8.3 Sponge bacteria interactions and the potential for bioremediation and biomonitoring

Sponges can offer a range of ecosystem services by retaining bacteria as indicators of water pollution and by remediating the bacterial pollution through consumption. These two processes are linked as both require sponge filtration and as the retention of bacteria both removes the bacteria from the environment and keeps it inside the sponge for detection (Stabili *et al.* 2008). Therefore, bioremediation and biomonitoring have not been separated in this discussion. To address bacterial pollution many studies have been carried out within a laboratory setting to establish the removal rates of bacteria. Regardless of the study, the same pattern whereby sponges concentrated bacteria, retaining them in a higher abundance than the ambient water has always been observed (De Goeij *et al.* 2008; Stabili *et al.* 2008; Topçu *et al.* 2010). Longo *et al.* (2010), for example, found that total faecal coliforms in seawater were 0.1 MPN $g^{-1}$ while the total faecal coliforms in sponges were 1.1 MPN $g^{-1}$ demonstrating the ability for sponges to concentrate bacteria from the water. This concentration effect of bacteria by sponges would also increase the chances of faecal indicator bacterial being detected, if sponges were used for biomonitoring or biomonitoring purposes.

Among the biomonitoring ability of sponges is the retention of coliforms from the environment as Longo *et al.* (2010) found a total of 1.5 MPN $g^{-1}$ of total coliforms in

sponges. This was higher than the number of faecal coliforms which were 1.1 MPN $g^{-1}$ but these values and retention ability were highly dependent on the health of the sponge, as starved sponges did not show any retention of these bacterial groups. This could indicate that sponges switch between a feeding and retention state depending on their nutrition. It could also mean that they can alternate between bioremediation and bioretention ability as both starved and unstarved sponges showed bioremediation, but only the unstarved sponges could be used for biomonitoring (Longo *et al.* 2010). Therefore, the nutritional status of the sponges needs to be understood before they can be used to sample bacteria.

The work of Stabili *et al.* (2008) also focused on the ability of sponges to detect microbial pollution using *Vibrio,* faecal coliforms and faecal streptococci abundance in sponges to compare polluted and less polluted areas. Samples of *Spongilla officinalis* living in proximity to a fish farm were compared to those from a site within a marine protected area. The fish farm contained 12 cages with sea bass at a stocking density of 60 fish per $m^3$. These sites were sampled at two different time periods, July and December 2005, and showed differences in the *Vibrio,* faecal coliforms and faecal streptococci over time and between sampling sites. The counts of viable *Vibrio* in sponge samples, for example, varied in the marine protected area with $1.6 \times 10^2 – 1.7 \times 10^3$ cfu $g^{-1}$, but remained stable at the fish farm between sampling periods with $1.2 \times 10^5$ cfu $g^{-1}$ from sponges 20 m below the fish farm (Stabili *et al.* 2008). This finding is indicative for biomonitoring as the fish farm management is likely to result in similar bacterial loading over time while the marine protected area will be affected by oceanic processes e.g. direction of the current which will affect the bacterial inputs and human activity such as shipping. In Dublin Bay, shipping resulted in significantly higher detection of *E. coli* and enterococci with *E. coli* increasing from 6 x MPN $ml^{-1}$ to 30 MPN $ml^{-1}$ due to sediment resuspension of the bacteria. As Stabili *et al.* (2008) sampled a nearshore marine protected area, similar factors could have affected the bacteria in their sponges at these sites, where bacteria may have been resuspended through water turbulence.

In addition to the biomonitoring application, there is also development in the use of sponges to remove bacterial pollution from aquaculture water before it is released into the environment (Milanese *et al.* 2003; Fu *et al.* 2006; Longo *et al.* 2016). This ability has been tested with a range of marine sponges including *Chondrilla nucula* and *Haliclona perelevis* (Milanese *et al.* 2003; Longo *et al.* 2016). The filtering experiments

of Milanese *et al.* (2003) found that *C. nucula* could remove 8 x $10^6$ cfu $ml^{-1}$ *E. coli* in 2 h but this quantity was the same after 7 h showing stagnation. This potentially indicated that sponges became saturated with the *E. coli* and so reduced their filtration slightly while they consumed some bacteria already in their tissue. *H. perelevis* has been studied more widely in an aquaculture setting and shows an ability to remove *Vibrio* sp., faecal streptococci and *E. coli* (Fu *et al.* 2006; Longo *et al.* 2016). This sponge reduced *E. coli* in the water from 8.3 x $10^6$ cfu $ml^{-1}$ to 2 x $10^4$ cfu $ml^{-1}$ in 10 h, offering significant bioremediation potential (Fu *et al.* 2006). Of these *E. coli* concentrations, 8 x $10^7$ cfu $g^1$ $h^{-1}$ were retained in the sponge tissue, indicating their ability to detect this bacteria pollution as well. Sponges are not the only type of filterfeedering organism which can be used for bioremediation as mussels demonstrate similar mechanisms (Longo *et al.* 2016). Longo *et al.* (2016) compared the bioremediation of *Vibrio* sp. and *E. coli* from the sponge *H. perelevis* and the mussel *Mytilus galloprovincialis*. They found the removal of these bacteria from the water had a maximal rate of $10^8$ and $10^5$ cfu $g^{-1}$ for sponges and mussels respectively (Longo *et al.* 2016). Therefore, sponges may be more effective at removing bacterial pollution than mussels. However, mussels only actively feed when their shell is open showing active and inactive periods each day, while sponges seem to filter continually (Wilson *et al.* 2005). This could have resulted in the lower retention observed by Longo et al (2016) in their mussels, as bacteria will not be retained, unless the organism is actively filtering thus affecting consumption and retention rate.

The level of bacteria consumption will also vary with species, as sponges are able to differentiate between food, symbiotic and infectious bacteria using NLR genes (Degnan 2015). This is likely to affect the rate of filtration of certain bacteria (Degnan 2015). This demonstrates that their filtering effect may not be as unselective as found by Wehrl *et al.* (2007). This could be related to the bacteria used by Wehrl *et al.* (2007) as although they factored in bacterial size and shape, they only tested the feeding ability to six bacteria including *Bacillus* and *Pseudomonas* which does not reflect the diversity that sponges will be exposed to. However, they did find differences in the filtration rate with symbiotic bacteria which were removed at a rate of 5 x $10^4$ cfu $g^{-1}$ $h^{-1}$ compared to 1-2 x $10^6$ cfu $g^{-1}$ $h^{-1}$ for consumed bacteria. This variation in filtration could possibly be linked to the NLR genes found by Degnan (2015). This will affect the bioremediation ability of sponges as they show differences in bacteria filtration rates and hence may not remove bacteria from the water at equal rates.

The bioretention and remediation ability is not only limited to bacteria and can include the removal of pollutants such as heavy metals and organic pollutants (Mahaut *et al.* 2013). The marine sponge *H. perelevis* can retain heavy metals including zinc and copper, and the polycyclic aromatic hydrocarbon fluoranthene at maximal concentrations of 2500 mg $kg^{-1}$, 330 mg $kg^{-1}$ and 430 µg $kg^{-1}$ respectively (Mahaut *et al.* 2013). This shows the potential for sponges to remove pollutants from the water and indicates that pollution events have occurred in that region. Mahaut *et al.* (2013) also found that concentrations of zinc, copper and fluoranthene were 20, 44 and 16 times higher than those retained in the mussel *Mytilus edulis*. Therefore, sponges not only retain bacteria more efficiently but also retain other aquatic pollutants at a higher concentration than mussels. In addition to filterfeeders, bioremediation can also be offered by bacteria and plants (Gifford *et al.* 2007). The nitrifying bacterium *Bacillus amyloliquefaciens*, for example, increased in abundance when high levels of ammonia were released into the water, subsequently bioremediating this pollution (Gad 2017). This process is likely to occur from a range of bacteria and to different pollutants as Gifford *et al.* (2007) noted that most bioremediation is from bacteria which themselves can become a pollutant. This adds to the complexity of detecting bacteria from the water with sponges as the bacteria detected could be from direct pollution or a bloom in bacteria to bioremediate a specific pollutant.

## 8.4 Integration of experimental work from this project

The unifying theme of this thesis was the interaction of freshwater sponges with bacteria, especially faecal indicator bacteria and those with antibiotic resistance. The experimental work has demonstrated how connected *E. fluviatilis* and *S. lacustris* are with bacteria including *E. coli* and *E. faecalis.* The experimental sections can also be linked to the three themes discussed above: ARB, sponge infection/defence and biomonitoring/bioremediation. Firstly, the sponges demonstrated the movement of aquatic bacteria from the water into their canal system. There is substantial evidence of the filterfeeding ability of sponges which can be used for sponge nourishment by phagocytosis of bacterial particles (Francis & Poirrier 1986; Vohmann *et al.* 2009; Longo *et al.* 2016). This very process resulted in the reduction/ bioremediation of aquatic bacteria as demonstrated in Chapter 3 using an antibiotic resistant *E. coli*. The fate of these bacteria was either retention for food or symbiosis which was detected in the sponges in Chapter 4 and 5. An abundance of bacteria has been detected in sponges with 32 - 3000 species found in each individual sponge (Thacker & Freeman 2012). The bacteria found in sponges included sponge specific bacteria which remain largely

unidentified to species level, and common aquatic bacteria including *Vibrio, Pseudomonas* and *Streptococci* (Stabili *et al.* 2008; Thacker & Freeman 2012). Although the relationship between sponges and *E. coli* has been widely explored mainly through bioremediation (Willenz *et al.* 1986; Stabili *et al.* 2008; Longo *et al.* 2010), the interaction with *E faecalis* does not appear to have been a focal point for research outside of this study. However, Velho-Pereira & Furtado (2014) found one species of enterococci associated with the marine sponge *Cinachya cavernosa*, but this group was not found in the other eight sponges tested. The authors noted that the isolation of enterococci was not expected as enterococci had not been reported in their offshore waters before, or in sponges. This indicates that enterococci are not common in marine sponges. In a marine environment, only coastal sponges are likely to be exposed to this genus of bacteria as they originate in the gastrointestinal tract of warm blooded animals and can enter the rivers and coastal areas from wastewater (Jett *et al.* 1994; Ghosh *et al.* 2011; Novais *et al.* 2013). However, they have not been associated with freshwater sponges which are likely to be exposed to these bacteria.

In Chapters 4 and 5, it was suggested that sponges were either able to prevent retention of *E. faecalis* or were infected by these bacteria reducing their filtering capabilities. If the sponge is preventing the retention of *E. faecalis*, this could be linked to the detection of LPS on these bacteria as demonstrated by Gardères *et al.* (2015) where the marine sponge *S. domuncula* was able to detect specific LPS from *Endozoicomonas, Pseudoalteromonas* and *E. coli* releasing macrophage genes as required to protect the sponges. This was demonstrated using Gram-negative bacteria, but it could also result in a reduced concentration of Gram-positive bacteria such as *E. faecalis* within the sponge. However, the evidence that live and dead sponges had similar numbers of *E. faecalis* implies that these bacteria can attach to the surface of sponges where potentially they could form a biofilm. Enterococci in a laboratory setting have shown the ability to form biofilm where cell communication resulted in aggregation of bacteria upon release of proteins including *asa1* and *cylA* (Daniels *et al.* 2011; Zheng *et al.* 2017). The aggregation of bacteria and release of pheromones can then bring bacteria into close proximity where they can potentially transfer genetic material through e.g. conjugal transfer (Cook *et al.* 2011). This could explain why transconjugant *E. faecalis* were isolated from the sponges in this study. As a wide variety of bacteria are found within sponges, the possibility of gene transfer is high. Bacteria isolated from sponges have genetic evidence of transduction including a COG0758 gene or conjugal transfer including the gene element COG3451

(Fan *et al.* 2012). However, the gene transfer evidence in isolates reported by Fan *et al.* (2012) could have occurred in the sponge or environment. Before the current study, conjugal transfer does not seem to have been experimentally demonstrated with sponges but may provide evidence that the HGT observed by Fan *et al.* (2012) occurred in the sponges. The gemmule surface was also coated in ARB (Chapter 6) and so this could also offer a potential site for gene transfer between bacteria.

The potential for bacteria in sponges to transfer resistance genes combined with the evidence of ARB present in the sponge and on the gemmules further elaborates on the points made by Wright (2010) and Marti *et al.* (2014) that aquatic ecosystems are reservoirs of ARB and their genes. If we are to minimise the risk of transfer of these ARB between the environment and clinic as suggested in Berendonk *et al.* (2015), mechanisms to reduce the transfer of these bacteria are needed. Sponges and other filterfeeders including mussels could offer this ecosystem service, as they remove bacterial pollution from the water by retention and filterfeeding (Longo *et al.* 2016). However, there is also the risk that these filterfeeders could facilitate the transfer of antibiotic resistance between bacteria which would also need to be removed. There is also potential for sponges to be used to sample for ARB as demonstrated by their ability to retain faecal indicator bacteria from rivers (Chapter 4). Selvin *et al.* (2009) found bacteria isolated from *F. cavernosa* including *Streptomyces*, *Pseudomonas* and *Vibrio* had plasmid-based resistance to antibiotics including erythromycin, ampicillin and oxytetracycline. Further sampling of sponges for ARB would help to provide quantification of the scale of ARB in the aquatic environment.

In addition to the sponge's ability to retain bacteria and remove it from the environment, there is also the potential for sponges and their associated bacteria to inhibit the growth of other microorganisms. This was demonstrated in Chapter 7 and also forms the basis of a growing research endeavour to isolate antimicrobial substances from sponges. Sponges can contain specific molecules such as halistanol-trisulphate which damages cell membranes before causing full lysis and death to the bacterium (Marinho *et al.* 2012). This molecule shows promise as a general antimicrobial compound. These bioactive compounds are primarily produced by the sponge to protect it from disease, but can also have human applications and help to reduce ARB in the environment within the sponge through cell lysis. Sponges also contain bacteria such as *Pseudomonas* which has antimicrobial effects against fungi and other bacteria (Keller-Costa *et al.* 2013). The

occurrence of *Pseudomonas* in the sponges, used in this study (Chapter 6), may indicate incorporation of these bacteria to protect the host. With the ability of sponges to detect bacteria specific LPS, the ability of sponges to selectively retain this bacteria seems likely (Gardères *et al.* 2015). These antimicrobial effects will impact on the biomonitoring, feeding, and conjugal transfer potential in the sponge or gemmule, thus highlighting the complexity of the host-bacteria interactions within sponges where many relationships are still unknown.

## 8.5 Novelty of presented studies and scope for future work

All the experimental chapters in this project had unique elements compared to the examined literature, however, some of the aspects may have been addressed in studies that were not easily available. The unique element of Chapter 3, the feeding trial, was the use of a fluorescence spectrometer to monitor the removal of planktonic bacteria by sponges. To further this work, the bacteria in the water would be stained e.g. with propidium iodide to quench fluorescence from dead bacteria. The trials would be repeated and hopefully remove background fluorescence from dead bacteria, thus validating these methods for detection of aquatic bacteria. However, potentially VBNC bacteria could still mean the fluorescence intensity and flow cytometry counts would be higher than on agar plate counts.

The novelty of Chapter 4 was related to the use of sponges as a biomonitor of microbial pollution in rivers. Sponges demonstrated the potential to sample faecal indicator bacteria in rivers while increasing the time-scale represented in the sample. It was also unique in testing retention related to different bacterial loads and time of exposure. Further work to develop sponges as a biomonitor for bacteria in rivers would involve laboratory studies to quantify the maximal bacteria retention of sponges, and genetics to ensure that faecal indicator bacteria do not form part of the symbiotic microbial community in sponges. The next step for field trials would involve either introducing surface-disinfected gemmule-grown sponges to monitor retention at specific times or to further test the effect of pollution source on bacteria retained by sponges. Sponges could also be developed as biomonitors for ARB.

Chapter 5 tested the suggestion from Lupo *et al.* (2012) that filterfeeders do facilitate the transfer of antibiotic resistance between bacteria. To further knowledge on the filtering effect of sponges for conjugal transfer in bacteria, this experiment should be repeated

with bacteria that are retained more efficiently by sponges. This experiment should also be tested with other filterfeeding organisms e.g. daphnia, as sponges were only one type of the available organisms for filterfeeders.

The unique element of Chapter 6 was the abundance of ARB on the gemmule surface. However, it did not find proof of bacteria inclusion inside the gemmule where future work should focus. Once the bacteria on the gemmule surface are removed, the gemmules could then be broken open for e.g. for DNA extraction and 16S PCR to detect if bacteria are present within the structure.

The novelty of the final experimental chapter was focusing on freshwater sponge extracts from different sites, and from adult and gemmule-grown sponges for inhibition to a variety of bacteria. For the antimicrobial properties of sponge extracts, it is necessary to further explore the effect of collection sites while quantifying total viable bacteria in the sponge and looking for pollutants in the sponges which could inhibit bacterial growth. Finally, the antimicrobial effect of sponge symbiotic bacteria and the chemical analysis of the most effective extract could also be tested.

Although this study has provided further knowledge on freshwater sponges and some of their interactions with bacteria, this research area requires more work. Research has not been found that addresses some simple sponge-microbe topics including the specific bacteria symbiotic with sponges, how sponge symbionts vary with location and if bacteria on the gemmule surface are from the adult sponge or the water. Sponges have the potential to facilitate the bacterial transfer of antibiotic resistance, so it is vital that there is a better understanding of the bacteria which are inside the sponges. This is particularly important as ARB have been isolated in this study and by other researchers from marine and freshwater sponges (Selvin *et al.* 2009; Keller-Costa *et al.* 2014; Eythorsdottir *et al.* 2016). As whole genomic sequencing becomes more available, this could help to address the questions posed above and could also address if bacteria are incorporated into the gemmule. On a more general note, it is mandatory that future studies continue to use higher replication to allow for the natural variation in sponges to be detected and prevent conclusions being drawn which would not hold under more rigorous testing. A standard method for calculating bacteria removal and quantity in sponges is also required.

# 9. Conclusion

Freshwater sponges showed high levels of interaction with bacteria both in a field and laboratory setting. Sponges fed on, retained and even inhibited the growth of bacteria. They also allowed for natural bacterial processes including the bacterial conjugal transfer of antibiotic resistance. The key findings of this study were:

- Sponges removed bacteria from water as they filtered.
- Sponge bacteria represented microbial water quality over a time-period of more than 24 h. Higher exposure to *E. faecalis* reduced the retention of bacteria in sponges.
- Sponges showed greater differences in bacteria abundance between river catchments than within one river suggesting that there may be merit in their use as a biomonitor for microbial water quality.
- The transfer of antibiotic resistance between bacteria occurred with live and dead sponges. More transconjugants were also found in the water when sponges were present.
- The gemmule surface was covered in bacteria including those with antibiotic resistance.
- Freshwater sponge extracts inhibited the growth of selected nosocomial bacteria. The antimicrobial effect was greater in adult sponges, but this varied greatly with collection site.

These have provided a further understanding of how freshwater sponges interact with bacteria during their seasonal lifecycle. This study also attempted to explore the natural variability between sponges by having higher replication than most published studies thus providing stronger evidence by taking account of observed variation. In conclusion, this project provided information which can be used to develop sponges as a biomonitor for microbial water quality. This will increase the detection period of aquatic bacteria beyond what is currently used with standard monitoring strategies relying on spot samples for water quality assessment. It also provided information on how bacteria in sponges reflected the proportion of bacterial loading. Bacterial conjugative transfer has been demonstrated with an aquatic organism, to add to the small number of studies on terrestrial species. Freshwater sponges contained ARB on their gemmule surface which was incorporated into the sponge upon hatching. Freshwater sponges also inhibited the growth of bacteria as a defence mechanism against infection; there may be potential for their bioactive compounds to be used in clinical applications to control microbial growth.

# 10. Appendix

Appendix 1. Location of sponges in Ireland

Selected catchments were searched for freshwater sponges. These were from Northern Ireland (Co. Antrim, Armagh, Fermanagh, Londonderry, and Tyrone) and Ireland (Co. Cavan and Monaghan). Proximity to lakes was used as a feature to identify potential locations as Stephens (1919) found maximal sponge abundance downstream of lakes due to stable water flow and food sources for the sponges. Co. Fermanagh and Co. Cavan form part of a lake district, so most sponges were found here (Table 10.1). Three of the five sponge species known to occur in Ireland were found: *E. fluviatilis*, *Ephydatia muelleri*, and *S. lacustris.* For the project, the main rivers for sponge collection were Cavan River, Downhill River, and Rag River. *E. fluviatilis* was not found in Downhill River until after repair work to the dam wall in March 2017. After this, it replaced *S. lacustris* throughout much of the channel.

Table 10.1. Rivers with sponges and identified species.

| **River** | **County** | **Species** |
|---|---|---|
| Bellatrain Lough outflow | Monaghan | *E. fluviatilis, E. muelleri* |
| Cavan River | Cavan | *E. fluviatilis* |
| Clonamullig Lough outflow | Cavan | *E. muelleri, S. lacustris* |
| Downhill River | Londonderry | *E. fluviatilis, S. lacustris* |
| Drumaa Lough outflow | Fermanagh | *S. lacustris* |
| Dromore River | Cavan | *E. muelleri* |
| Finn River | Fermanagh | *E. fluviatilis* |
| Killywilly Lough outflow | Cavan | *S. lacustris* |
| Lough Bawn outflow | Monaghan | *E. fluviatilis, S. lacustris* |
| Lough Erne (Manor house) | Fermanagh | *S. lacustris* |
| Lough Neagh (Brockagh, Curran Quay) | Tyrone | *E. muelleri* |
| Lough Neagh (Kinnego Bay) | Armagh | *Ephydatia* sp. |
| Orritor River | Tyrone | *E. fluviatilis, S. lacustris* |
| Rag River | Cavan | *E. fluviatilis, E. muelleri, S. lacustris* |
| River Maine | Antrim | *E. fluviatilis* |
| Six Mile Water | Antrim | Unidentifiable to species level |

Appendix 2. How to hatch sponges

Sponges used for experiments throughout this study were hatched from gemmules with disinfected surfaces. Gemmules were collected during autumn and winter when the adult sponge die back into this resting phase. On collection gemmules were treated with 1% $H_2O_2$ for 10 minutes, adapted from Rasmont (1970). The gemmules were rinsed in autoclaved water before being centrifuged at 30,000 rpm for 5 min to draw the gemmules to the bottom of the tube. The gemmules were rinsed another three times before storage in autoclaved water at 4 ºC until needed. The water was changed once a month with fresh sterile water.

*Hatching water and vessel*

Initial hatching of gemmules (tested on *S. lacustris*) was attempted in autoclaved water (river water, water with added salts, or mineral water), but these was unsuccessful. UV treated mineral water (10 minutes at 254 nm), however, did result in hatching, so this was used for all experiments. It was also found that sponges could be hatched on a range of surfaces including plastics and glass, but the hatching rate varied with the surface from 91% on Eppendorf tubes to 38% in microplate wells.

*Variation with species and batch*

The hatching rate not only varied with the surface, but also with sponge species and batch. *E. fluviatilis* gemmules showed higher hatching rates than *S. lacustris* gemmules. The *S. lacustris* gemmules varied in hatch rate from 18–91% depending on batch while the hatch rate of *E. fluviatilis* was 85–95% regardless of which batch was used.

*Hatching temperature and microbes in gemmules*

To maximise gemmule hatching for sponges collected from Irish waterbodies, a suitable temperature for the sponges needed to be found. *S. lacustris* gemmules were hatched in 12 welled sterile microplates with 4 ml of UV treated mineral water at 10 ºC, 15 ºC, 20 ºC, 25 ºC and 30 ºC. The hatching rate and sponge size were measured. After the maximum sponge size was reached, randomly selected sponges from each temperature were placed on nutrient agar and incubated at their hatching temperature for up to 72 h before counting. Where the numbers of colonies exceeded 400, they were estimated to the nearest 50 colonies. No differentiation was made between types of growth, or between fungal and bacterial colonies. The water from selected wells on the control plates (water only) was also plated and incubated at each temperature.

The hatching rate of the sponges was highest for 25 ºC but only slightly lower at 15 ºC (Table 10.2). None of the gemmules hatched at 30 ºC and they were killed by this temperature, turning black. Active *S. lacustris* have been found in Europe at 27°C (Økland & Økland 1996), but in North American, unlike the Irish gemmules, they survive at 34 ºC (Harrison 1974). Hatching was fastest at 20 ºC or 25 ºC, and the subsequent growth rate increased with temperature (Table 10.3). Once maximum size was reached the sponges started to shrink again before death. Shrinking started to occur quicker at higher temperatures size due to higher biological demand. Therefore, 20 ºC was chosen as it allowed for rapid growth, but sponge survival was maintained for the length of time required for experiments.

Table 10.2. Percentage hatch for gemmules in different temperature incubators and the number of days required for hatching.

| Incubation temperature (ºC) | Hatch rate (%) | Hatch time (days) |
|---|---|---|
| 10 | 29 | 4-21 |
| 15 | 46 | 7-10 |
| 20 | 35 | 4-7 |
| 25 | 48 | 4-7 |
| 30 | 0 | N/A |

Table 10.3. Sponge area (arithmetic mean, minimum and maximum) and the growth rate before the sponges reached maximal size where SE = Standard Error, Min = minimum, Max = maximum.

| | Sponge area ($mm^2$) | | | Days to reach max size | Growth rate ($mm^2$ $day^{-1}$) |
|---|---|---|---|---|---|
| Incubation temperature (°C) | Mean ± SE | Min | Max | Mean ± SE | Mean ± SE |
| 10 | 2.21 ± 0.89 | 1.31 | 3.10 | 11 ± 2 | 0.07 ± 0.01 |
| 15 | 4.53 ± 1.07 | 1.53 | 8.80 | 8 ± 2 | 0.31 ± 0.21 |
| 20 | 3.21 ± 0.51 | 2.22 | 4.59 | 6 ± 1 | 0.30 ± 0.04 |
| 25 | 3.04 ± 0.45 | 1.39 | 4.57 | 4 ± 1 | 0.36 ± 0.11 |

The microbial analysis on selected wells from each microplate showed varying levels of growth (Table 10.4). The control plates at each incubator temperature showed no growth on agar plates, therefore any bacteria which did grow were either present on or in the gemmule/sponge. Some unhatched gemmules were plated and these had fewer than 3 colonies indicating that most bacteria present had originally been inside the gemmule

(however, this was not found in Chapter 6). All the sponges plated had bacteria and/or fungi present on or in them (Table 4). The range of total viable counts showed high variability across the tested temperature range but was lowest and least variable at 20 ºC further indicating this temperature represented the optimal choice for experiments.

Table 10.4. Range in microbial colony counts from sponges hatched at different temperatures where TVC = total viable count.

| Incubation temperature (ºC) | TVC range |
|---|---|
| 10 | 330 – 700 |
| 15 | 200 - >1000 |
| 20 | 58 – 331 |
| 25 | 39 - >1000 |

Appendix 3. Supplementary data for chapter 4 – biomonitoring of microbial water quality in freshwater sponges

Freshwater sponge samples were collected from Orritor, Cavan and Rag river for the quantification of coliform and enterococci present in sponges (see figure 4.6a and figure 4.6b). When the sponges were collected, a single water sample was also collected into a sterile bottle from the middle of each river channel. The coliforms and enterococci in the water samples were quantified by plating water onto MacConkey and Slanetz & Bartley media respectively. 1 ml of water from each sample was plated for the undiluted sample. Tenfold serial dilutions were also plated to allow for quantification of the bacteria. The bacteria counts were averaged over three replicates at the quantification dilution.

The river with the highest abundance of both coliforms and enterococci was Cavan River. Orritor and Rag river had similar low abundances of these two bacterial groups. The sponges contained 10-800 times the quantity of coliforms to that found in the collected water sample (Figure 4.6a). The same pattern was found with the enterococci where sponges contained 3-10 times more enterococci than the water sample. Therefore, the sponges concentrate the bacteria from the water for retention in their bodies which can be enumerated with sampling.

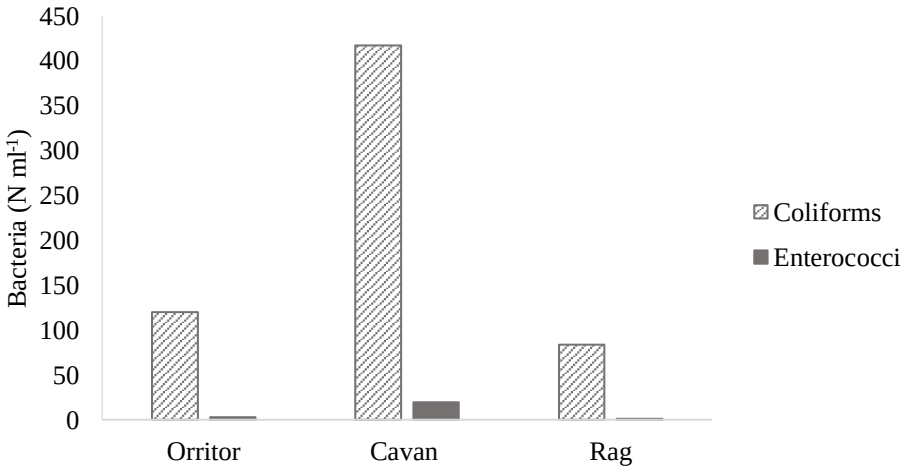



Figure 10.1. Arithmetic mean values for the coliforms and enterococci in the water from three rivers.

Appendix 4. Supplementary data for chapter 5 – do freshwater sponges facilitate the transfer of antibiotic resistance in waterborne *Enterococcus faecalis*?

A number of conjugation trials were completed with the sponges using a variety of vessels (Table 10.5). These tests were used to increase the efficiency of the experiment and to maximise the chances of retrieving transconjugants from the sponges. Initially experiments were on sponges grown in microplates before Eppendorf tubes were used. The experiment was upscaled to universal tubes before the use of glass petri dishes (Chapter 5). In total conjugation experiments were tested with 271 sponges (dead or alive, *E. fluviatilis* or *S. lacustris*) and transconjugants were isolated from 256 of these sponges.

Table 10.5. Summary of conjugation experiments completed with sponges in different vessels and the reason for using the experiment design

| | | | **Transconjugant number** | | |
|---|---|---|---|---|---|
| **Vessel** | **Sponge species** | **Number tested** | **Range** | **Mean** | **Reason for use** |
| Microplate | *S. lacustris* | 4 live | 1-10 | 4 | First experiment to test feasibility |
| Eppendorf tubes | *S. lacustris* | 99 live | 0-29 | 2 | Tubes allowed for sponges to be individualised |
| Universal tubes | *S. lacustris*<br><br>*E. fluviatilis* | 20 live<br>24 dead<br>20 live<br>20 dead | 6-39<br>2-40<br>0-219<br>0-34 | 18<br>14<br>28<br>16 | Higher volume of water for sponge to filter. Dead sponges would act as control to sponge filtering |
| Glass petri dishes | *E. fluviatilis* | 42 live<br>42 dead | 0-16<br>2-32 | 5<br>8 | Bacteria stuck to glass surface less than plastic. *E. fluviatilis* had higher culture success and both sponge species showed similar pattern |

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